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<input type="checkbox"/> Additional inventors are being named on page 2 attached hereto					
TITLE OF THE INVENTION (280 characters max)					
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ENZYME INHIBITION

The present invention relates to enzyme inhibition, and in particular to the inhibition of an enzyme that we call asparaginyl endopeptidase which we
5 have found is involved in processing antigens, particularly microbial antigens, by the immune system.

The immune response to protein antigens involves a large number of individual gene products, and new ones are still being discovered. In
10 some cases the gene products were known *per se*, but not known to be involved in the immune response.

An immune response may be raised towards foreign antigens, for example, antigens associated with microorganisms, or the immune
15 response may be caused by a response to a self antigen (autoimmunity). In either case, there exists the need for methods and means for modulating the immune system and how it responds to these foreign and self antigens.

Because proteins must be proteolytically processed (ie partially degraded)
20 before the T cells of the immune system can respond, one set of proteins of importance in an immune response are proteolytic enzymes (proteases). It is typically proteolysed proteins (ie protein fragments) which are presented by antigen presenting cells on MHC Class I or Class II molecules.

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Several different aspartic and cysteine proteases are thought to be involved in invariant chain and antigen processing (see, for example, Fineschi & Miller (1997) *Trends Biochem. Sci.* 22, 377-382; and Chapman (1998)

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Curr. Op. Immunol. 10, 93-102). For example, there is now good evidence that cathepsin S plays a role in the final stages of invariant chain processing (see, for example, Riese *et al* (1996) *Immunity* 4, 357-366; and Villadangos *et al* (1997) *J. Exp. Med.* 186, 549-560).

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WO 97/40066 relates to the suppression of immune response by inhibiting cathepsin S.

We have now found, surprisingly, a further protease which is involved in processing protein antigens. The enzyme has asparaginyl endopeptidase (AEP) activity and, until the work described in this patent application, was not known to be involved in the immune response.

An enzyme with AEP activity was first detected in legumes and was called "legumain". Enzymes with AEP activity have also been detected in the blood fluke *Schistosoma mansoni* where it is involved in degrading haemoglobin (haemoglobinase) and, more recently, in mammals from which the cDNA has also been cloned (Chen *et al* (1997) *J. Biol. Chem.* 272, 8090-8096). For the avoidance of doubt, and in the event that different names are used, the animal enzymes with asparaginylendopeptidase activity may interchangeably be referred to as "asparaginyl endopeptidase", "AEP", "legumain" or "haemoglobinase". It will be appreciated that any particular animal, such as a human, may have more than one enzyme with asparaginyl endopeptidase activity, and that, in the context of the invention now described, an inhibitor of AEP may inhibit one or more forms of AEP.

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25 An enzyme with AEP activity is readily able to cleave the substrate Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide to release a fluorescent product, where Z is benzyloxy carbonyl.

By an "inhibitor of asparaginyl endopetidase" we include any suitable inhibitor. The inhibitor may be an enzyme inhibitor such as a competitive inhibitor or it may be a non-competitive inhibitor. The term also includes inhibitors of the provision of an active form of AEP in a cell such as
5 inhibitors which inhibit the transcription of an AEP gene or the processing or translation of an AEP mRNA. As discussed below, suitable such inhibitors include AEP-selective antisense agents.

AEP is a cysteine protease and so inhibitors which chemically react with
10 the active cysteine residue are suitable and are, typically, irreversible inhibitors. Other amino acid residues, such as histidine, may be present at the active site and inhibitors which react with any active site residue are suitable.

15 In one embodiment of the invention the AEP inhibitor is a competitive inhibitor. Typically, the competitive inhibitor is a peptide comprising an asparagine-containing peptide. Suitably, the peptide is a peptide comprising a known AEP cleavage site but which, because of its affinity for AEP can compete for another AEP cleavage site and substantially
20 prevent cleavage at the said other cleavage site if present in sufficient concentration. Known cleavage sites for AEP in tetanus toxin are described in Chen *et al* (1997) *J. Biol. Chem.* 272, 8090-8098.

In a preferred embodiment of the invention the competitive inhibitor has
25 the general structure $(X_1)_p N(X_2)_q$ wherein X_1 and X_2 are amino acid residues, N is an asparagine residue, p is 3 to 6 and q is 1 to 3.

Suitably, the competitive inhibitor is a peptide which comprises the peptide sequence Ala-Glu-Asn-Lys or, less preferably, Lys-Asn-Asn-Glu. Preferably, the competitive inhibitor is an N and C-terminal blocked peptide Ala-Glu-Asn-LysNH (AENK) or, less preferably, Lys-Asn-Asn-Glu-NH (KNNE).

As noted, and as is shown in Example 2, the class II MHC invariant chain is a substrate for AEP. Inspection of the invariant chain sequence indicates that any of the following asparagine residues may be processed by AEP: 71, 76, 131, 148 and 154 found in both p31 and p41 isoforms (p31 and p41 are two alternatively spliced forms of the invariant chain), and 217, 219, 238, 244 and 252 found only in the p41 isoform.

The sequence of the human invariant chain is given below. The p41 specific region is underlined.

MHRRRSRSCREDQKPVMDQDRLISNNEQLPMLGRRPGAPESKCSRGAlyT
GFSILVTLLLAGQATTAYFLYQQQGRLDKLTVTSQNLQLENLRMKLPKPPK
PVSKMRMATPLLMOALPMGALPQGPMQNATKYGNMTEDHVMHLLQNADPLK
VY?PLKGSFPENLTHLKNTMETIDWKVFESWMHWWLLFEMSRHSLEQKPTD
APPKVLTKCQEEVSHIPAVHPGSFRPKCDENGNYLPQCYGSIGYCWCVFP
NGTEVPNTRSRGHHNCSESLELEDPSSGLGVTKQDLGPVPM

The complete sequence of the mRNA (and encoded polypeptide) for the HLA-DR-associated invariant chain is described in Strubin *et al* (1984) *EMBO J.* 3, 869-872. The structure of the human Ia-associated invariant (gamma)-chain is described in O'Sullivan *et al* (1986) *Proc. Natl. Acad. Sci. USA* 83, 4484-4488. Kudo *et al* (1985) *Nucl. Acids Res.* 13, 8827-8841 describes the structure of the human gene encoding the invariant gamma chain of class II histocompatibility antigens.

Competitive inhibitors may be made based on the sequences which comprise, for example, two or three residues in each direction from the asparagine residue. Thus, for example, competitive inhibitors include
5 residues 69 to 73, 68 to 74, 74 to 78, 73 to 79, 129 to 133, 128 to 134 and so on of the invariant chain. As is described in Example 2, both the p31 and p41 forms of the invariant chain are cleaved by AEP.

Peptides may be synthesised by the Fmoc-polyamide mode of solid-phase
10 peptide synthesis as disclosed by Lu *et al* (1981) *J. Org. Chem.* 46, 3433 and references therein. Temporary N-amino group protection is afforded by the 9-fluorenylmethyloxycarbonyl (Fmoc) group. Repetitive cleavage of this highly base-labile protecting group is effected using 20% piperidine in N,N-dimethylformamide. Side-chain functionalities may be protected as
15 their butyl ethers (in the case of serine threonine and tyrosine), butyl esters (in the case of glutamic acid and aspartic acid), butyloxycarbonyl derivative (in the case of lysine and histidine), trityl derivative (in the case of cysteine) and 4-methoxy-2,3,6-trimethylbenzenesulphonyl derivative (in the case of arginine). Where glutamine or asparagine are C-terminal residues, use is
20 made of the 4,4'-dimethoxybenzhydryl group for protection of the side chain amido functionalities. The solid-phase support is based on a polydimethyl-acrylamide polymer constituted from the three monomers dimethylacrylamide (backbone-monomer), bisacryloylethylene diamine (cross linker) and acryloylsarcosine methyl ester (functionalising agent).
25 The peptide-to-resin cleavable linked agent used is the acid-labile 4-hydroxymethyl-phenoxyacetic acid derivative. All amino acid derivatives are added as their preformed symmetrical anhydride derivatives with the exception of asparagine and glutamine, which are added using a reversed

N,N-dicyclohexyl-carbodiimide/1-hydroxybenzotriazole mediated coupling procedur . All coupling and deprotection reactions are monitored using ninhydrin, trinitrobenzene sulphonic acid or isotin test procedures. Upon completion of synthesis, peptides are cleaved from the resin support with
5 concomitant removal of side-chain protecting groups by treatment with 95% trifluoroacetic acid containing a 50% scavenger mix. Scavengers commonly used are ethanedithiol, phenol, anisole and water, the exact choice depending on the constituent amino acids of the peptide being synthesised. Trifluoroacetic acid is removed by evaporation *in vacuo*, with
10 subsequent trituration with diethyl ether affording the crude peptide. Any scavengers present are removed by a simple extraction procedure which on lyophilisation of the aqueous phase affords the crude peptide free of scavengers. Reagents for peptide synthesis are generally available from Calbiochem-Novabiochem (UK) Ltd, Nottingham NG7 2QJ, UK.
15 Purification may be effected by any one, or a combination of, techniques such as size exclusion chromatography, ion-exchange chromatography and (principally) reverse-phase high performance liquid chromatography. Analysis of peptides may be carried out using thin layer chromatography, reverse-phase high performance liquid chromatography, amino-acid analysis
20 after acid hydrolysis and by fast atom bombardment (FAB) mass spectrometric analysis.

In a further embodiment, the inhibitor may be a non-competitive or irreversible inhibitor. AEP activity can be blocked by high concentrations
25 of inhibitors which block all cysteine proteases and some serine proteases. However, it is particularly preferred that the inhibitors are selective for AEP. Non-competitive (irreversible or covalent) inhibitors are more preferred than competitive inhibitors.

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naturally occurring amino acid residues or they may be residues which have non-natural side chains.

The peptide sequence before the C terminal asparagine and active group
5 may be any suitable peptide sequence, but it will be appreciated that it is the sequence at a known AEP cleavage site.

As noted, AEP cleaves both the p31 and p41 isoforms of the invariant chain. Non-competitive (or irreversible) inhibitors may readily be
10 designed based on the residues N-terminal to the cleaved asparagine. As described above, any of the following asparagine residues may be processed by AEP: 71, 76, 131, 148 and 151 found in both p31 and p41, and 217, 219, 238, 244 and 252 found only in the p41 isoform. Thus, non-competitive inhibitors may be made which contain some of the amino
15 acid residues N-terminal to these particular sites wherein the asparagine residue is linked to a group (Q) capable of reacting with the active site cysteine or other active site residue of AEP and, conveniently, forming a covalent complex thereby eliminating catalytic activity. Thus, AEP inhibitors may include BI-Ser-Gln-Asn-Q; BI-Leu-Glu-Asn-Q; BI-Leu-Gln-Asn-Q; BI-Pro-Glu-Asn-Q; BI-Leu-Lys-Asn-Q (corresponding to
20 asparagines 71, 76, 131, 148 and 154).

Two preferred inhibitors are BI-Gln-Asn-Q and BI-Glu-Asn-Q.

25 Other AEP inhibitors may include BI-Asp-Glu-Asn-Q; BI-Asn-Gly-Asn-Q; BI-Phe-Pro-Asn-Q; BI-Val-Pro-Asn-Q; and BI-His-His-Asn-Q. These inhibitors relate to Asn residues of the p41 isoform with numbers 217, 219, 238, 244 and 252, respectively.

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Typically, peptide aldehyde inhibitors have the structure:

Acetyl (or other blocking group)-X_n-Asparaginal

where X is an amino acid residue and n is between 1 and 100, preferably between 1 and 50, more preferably between 1 and 10 and typically 2 to 6.

A suitable peptide aldehyde includes acetyl-alanyl-glutamyl-asparaginal.

Elastatinal blocks AEP activity; however a more specific variant, in which the C terminal amino residue is replaced by asparagine, may be useful.

Figure 4 shows the structures of some useful inhibitors.

Typically, peptide chloromethylketone inhibitors have the structure.

Acetyl (or other blocking group)-X_n-asparaginyl-chloromethyl ketone
where X and n are as above.

A suitable peptide includes acetyl-alanyl-glutamyl-asparaginyl-chloromethylketone and acetyl-tyrosyl-valyl-alanyl-asparaginyl-chloromethylketone (analogous to ICE protease inhibitor YVAD-cmk); see Figure 4 for further details.

Typically, peptide fluoromethylketone inhibitors have analogous structures to peptide chloromethylketone inhibitors except for the replacement of a chloro group with a fluoro group.

Typically, peptide diazomethane inhibitors have the structure



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where Bl is any suitable blocking group including acetyl or benzyloxycarbonyl and X and n are as above. A diazomethane on the C-terminus of a peptide has a general structure $\text{R}-\text{C}(\text{O})\text{CHN}_2$ where R represents the peptide.

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A suitable peptide includes Bl-alanyl-asparaginyl-diazomethane or Bl-alanyl-glutamyl-asparaginyl-diazomethane.

Typically, peptide vinyl sulphone inhibitors have the structure

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where Bl is an N-terminal blocking group such as acetyl or benzyloxycarbonyl, X and n are as above, and R is any suitable alkyl or aryl terminating group and includes C_1 to C_{10} alkyl, phenyl, benzyl, naphthyl and the like.

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A suitable peptide includes morpholinurea-leucyl-asparaginyl-vinyl sulphone-phenyl or morpholinurea-alanyl-glutamyl-asparaginyl-vinyl sulphone-phenyl.

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Other suitable peptides include peptidyl(acyloxy) methanes and peptidyl N,O-diacylhydroxamates.

It will be appreciated from the foregoing that AEP inhibitors may be designed based on other cysteine protease inhibitors such as E-64 (1-trans-epoxysuccinyl-leucylamide (4-guanido)-butane). Leupeptin (acetyl-leucyl-
5 leucyl-arginal), Antipain ([[(S)-1-carboxy-2-phenyl]-carbamoyl-Arg-Val-arginal), Elastinal (N-[(S)-1-carboxy-isopentyl]-carbamoyl- α -(2-imino-hexahydro-4(S)-pyrimidyl)-L-glycyl-L-glutaminy-L-alaninal), TLCK (tosyllysylchloromethylketone) and TPCK (tosylphenylalanylchloromethylketone), for example by introduction of an
10 asparaginyl residue at an appropriate place in place of an existing amino acid residue.

Vinyl sulphone inhibitors of cysteine proteases are described in Palmer *et al* (1995) *J. Med. Chem.* 38, 3193-3196. Peptide aldehyde inhibitors of
15 cysteine proteases are described in Thomson *Methods Enzymol.* 46, 220 - and Vinitzky *et al* (1994) *J. Biol. Chem.* 269, 29860-29866 639-648. Peptide diazomethanes as inhibitors of cysteine proteases are described in Shaw (1994) *Methods Enzymol.* 244, 649-656 and Shaw & Green (1981) *Methods Enzymol.* 80, 820-826. Peptide (aryloxy) methanes as inhibitors
20 of cysteine proteases are described in Krantz (1994) *Methods Enzymol.* 244, 656-671. Peptide N,O-diarylhydroxamates as inhibitors of cysteine proteases are described in Brömme & Demuth (1994) *Methods Enzymol.* 244, 671-685. Peptide chloromethyl ketones as inhibitors of cysteine proteases are described in Williams & Mann (1993) *Methods Enzymol.*
25 222, 503-513. Thus, methods for synthesising suitable inhibitors are well known to the person skilled in the art.

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Antisense oligonucleotides are single-stranded nucleic acid, which can specifically bind to a complementary nucleic acid sequence. By binding to the appropriate target sequence, an RNA-RNA, a DNA-DNA, or RNA-DNA duplex is formed. These nucleic acids are often termed "antisense" because they are complementary to the sense or coding strand of the gene. Recently, formation of a triple helix has proven possible where the oligonucleotide is bound to a DNA duplex. It was found that oligonucleotides could recognise sequences in the major groove of the

DNA double helix. A triple helix was formed thereby. This suggests that it is possible to synthesise a sequence-specific molecules which specifically bind double-stranded DNA *via* recognition of major groove hydrogen binding sites.

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By binding to the target nucleic acid, the above oligonucleotides can inhibit the function of the target nucleic acid. This could, for example, be a result of blocking the transcription, processing, poly(A) addition, replication, translation, or promoting inhibitory mechanisms of the cells, such as promoting RNA degradations.

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Antisense oligonucleotides are prepared in the laboratory and then introduced into cells, for example by microinjection or uptake from the cell culture medium into the cells, or they are expressed in cells after
15 transfection with plasmids or retroviruses or other vectors carrying an antisense gene. Antisense oligonucleotides were first discovered to inhibit viral replication or expression in cell culture for Rous sarcoma virus, vesicular stomatitis virus, herpes simplex virus type 1, simian virus and influenza virus. Since then, inhibition of mRNA translation by antisense
20 oligonucleotides has been studied extensively in cell-free systems including rabbit reticulocyte lysates and wheat germ extracts. Inhibition of viral function by antisense oligonucleotides has been demonstrated *in vitro* using oligonucleotides which were complementary to the AIDS HIV retrovirus RNA (Goodchild, J. 1988 "Inhibition of Human
25 Immunodeficiency Virus Replication by Antisense Oligodeoxynucleotides", *Proc. Natl. Acad. Sci. (USA)* 85(15), 5507-11). The Goodchild study showed that oligonucleotides that were most effective were complementary to the poly(A) signal; also effective were those

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A detailed description of the H-phosphonate approach to synthesizing oligonucleoside phosphorothioates is provided in Agrawal and Tang (1990) *Tetrahedron Letters* 31, 7541-7544, the teachings of which are hereby
5 incorporated herein by reference. Syntheses of oligonucleoside methylphosphonates, phosphorodithioates, phosphoramidates, phosphate esters, bridged phosphoramidates and bridge phosphorothioates are known in the art. See, for example, Agrawal and Goodchild (1987) *Tetrahedron Letters* 28, 3539; Nielsen *et al* (1988) *Tetrahedron Letters* 29, 2911; Jager
10 *et al* (1988) *Biochemistry* 27, 7237; Uznanski *et al* (1987) *Tetrahedron Letters* 28, 3401; Bamwarth (1988) *Helv. Chim. Acta.* 71, 1517; Crosstick and Vyle (1989) *Tetrahedron Letters* 30, 4693; Agrawal *et al* (1990) *Proc. Natl. Acad. Sci. USA* 87, 1401-1405, the teachings of which are incorporated herein by reference. Other methods for synthesis or
15 production also are possible. In a preferred embodiment the oligonucleotide is a deoxyribonucleic acid (DNA), although ribonucleic acid (RNA) sequences may also be synthesized and applied.

The oligonucleotides useful in the invention preferably are designed to resist
20 degradation by endogenous nucleolytic enzymes. *In vivo* degradation of oligonucleotides produces oligonucleotide breakdown products of reduced length. Such breakdown products are more likely to engage in non-specific hybridization and are less likely to be effective, relative to their full-length counterparts. Thus, it is desirable to use oligonucleotides that are resistant
25 to degradation in the body and which are able to reach the targeted cells. The present oligonucleotides can be rendered more resistant to degradation *in vivo* by substituting one or more internal artificial internucleotide linkages for the native phosphodiester linkages, for example, by replacing phosphate

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with sulphur in the linkage. Examples of linkages that may be used include phosphorothioates, methylphosphonates, sulphone, sulphate, keryl, phosphorodithioates, various phosphoramidates, phosphate esters, bridged phosphorothioates and bridged phosphoramidates. Such examples are
5 illustrative, rather than limiting, since other internucleotide linkages are known in the art. See, for example, Cohen, (1990) *Trends in Biotechnology*. The synthesis of oligonucleotides having one or more of these linkages substituted for the phosphodiester internucleotide linkages is well known in the art, including synthetic pathways for producing
10 oligonucleotides having mixed internucleotide linkages.

Oligonucleotides can be made resistant to extension by endogenous enzymes by "capping" or incorporating similar groups on the 5' or 3' terminal nucleotides. A reagent for capping is commercially available as Amino-
15 Link II™ from Applied BioSystems Inc, Foster City, CA. Methods for capping are described, for example, by Shaw *et al* (1991) *Nucleic Acids Res.* 19, 747-750 and Agrawal *et al* (1991) *Proc. Natl. Acad. Sci. USA* 88(17), 7595-7599, the teachings of which are hereby incorporated herein by reference.

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A further method of making oligonucleotides resistant to nuclease attack is for them to be "self-stabilized" as described by Tang *et al* (1993) *Nucl. Acids Res.* 21, 2729-2735 incorporated herein by reference. Self-stabilized oligonucleotides have hairpin loop structures at their 3' ends, and show
25 increased resistance to degradation by snake venom phosphodiesterase, DNA polymerase I and fetal bovine serum. The self-stabilized region of the oligonucleotide does not interfere in hybridization with complementary nucleic acids, and pharmacokinetic and stability studies in mice have shown

increased *in vivo* persistence of self-stabilized oligonucleotides with respect to their linear counterparts.

In accordance with the invention, the inherent binding specificity of antisense oligonucleotides characteristic of base pairing is enhanced by limiting the availability of the antisense compound to its intend locus *in vivo*, permitting lower dosages to be used and minimizing systemic effects. Thus, oligonucleotides are applied locally to achieve the desired effect. The concentration of the oligonucleotides at the desired locus is much higher than if the oligonucleotides were administered systemically, and the therapeutic effect can be achieved using a significantly lower total amount. The local high concentration of oligonucleotides enhances penetration of the targeted cells and effectively blocks translation of the target nucleic acid sequences.

The oligonucleotides can be delivered to the locus by any means appropriate for localized administration of a drug. For example, a solution of the oligonucleotides can be injected directly to the site or can be delivered by infusion using an infusion pump. The oligonucleotides also can be incorporated into an implantable device which when placed at the desired site, permits the oligonucleotides to be released into the surrounding locus.

The oligonucleotides may be administered *via* a hydrogel material. The hydrogel is noninflammatory and biodegradable. Many such materials now are known, including those made from natural and synthetic polymers. In a preferred embodiment, the method exploits a hydrogel which is liquid below body temperature but gels to form a shape-retaining semisolid hydrogel at or near body temperature. Preferred hydrogel are polymers of ethylene oxide-

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propylene oxide repeating units. The properties of the polymer are dependent on the molecular weight of the polymer and the relative percentage of polyethylene oxide and polypropylene oxide in the polymer. Preferred hydrogels contain from about 10 to about 80% by weight ethylene oxide and from about 20 to about 90% by weight propylene oxide. A particularly preferred hydrogel contains about 70% polyethylene oxide and 30% polypropylene oxide. Hydrogels which can be used are available, for example, from BASF Corp., Parsippany, NJ, under the tradename Pluronic^R.

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In this embodiment, the hydrogel is cooled to a liquid state and the oligonucleotides are admixed into the liquid to a concentration of about 1 mg oligonucleotide per gram of hydrogel. The resulting mixture then is applied onto the surface to be treated, for example by spraying or painting during surgery or using a catheter or endoscopic procedures. As the polymer warms, it solidifies to form a gel, and the oligonucleotides diffuse out of the gel into the surrounding cells over a period of time defined by the exact composition of the gel.

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The oligonucleotides can be administered by means of other implants that are commercially available or described in the scientific literature, including liposomes, microcapsules and implantable devices. For example, implants made of biodegradable materials such as polyanhydrides, polyorthoesters, polylactic acid and polyglycolic acid and copolymers thereof, collagen, and protein polymers, or non-biodegradable materials such as ethylenevinyl acetate (EVAc), polyvinyl acetate, ethylene vinyl alcohol, and derivatives thereof can be used to locally deliver the oligonucleotides. The oligonucleotides can be incorporated into the material as it is polymerized or

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solidified, using melt or solvent evaporation techniques, or mechanically mixed with the material. In one embodiment, the oligonucleotides are mixed into or applied onto coatings for implantable devices such as dextran coated silica beads, stents, or catheters.

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The dose of oligonucleotides is dependent on the size of the oligonucleotides and the purpose for which is it administered. In general, the range is calculated based on the surface area of tissue to be treated. The effective dose of oligonucleotide is somewhat dependent on the length and chemical composition of the oligonucleotide but is generally in the range of about 30
10 to 3000 μg per square centimetre of tissue surface area.

The oligonucleotides may be administered to the patient systemically for both therapeutic and prophylactic purposes. The oligonucleotides may be
15 administered by any effective method, for example, parenterally (eg intravenously, subcutaneously, intramuscularly) or by oral, nasal or other means which permit the oligonucleotides to access and circulate in the patient's bloodstream. Oligonucleotides administered systemically preferably are given in addition to locally administered oligonucleotides, but
20 also have utility in the absence of local administration. A dosage in the range of from about 0.1 to about 10 grams per administration to an adult human generally will be effective for this purpose.

AEP-selective antisense agents, such as antisense oligonucleotides, may be
25 designed and made by the person skilled in the art by reference, for example, to the cDNA encoding AEP which is described in Chen *et al* (1997) *J. Biol. Chem.* 272, 8090-8098, incorporated herein by reference. It will be appreciated that there may be more than one AEP gene or that an

AEP gene can give rise to more than one AEP transcript. Typically, the antisense oligonucleotide has at least 10 bases and, more preferably, at least 15 bases. Typically, the antisense oligonucleotides has around 18 to 20 bases. Although oligonucleotides may be designed and made which are
5 complementary to any region of an AEP gene or mRNA transcript, in preferred embodiments the antisense oligonucleotides correspond to the region of the mRNA which contains the translation initiation signals or which encodes the N-terminus of AEP polypeptide. In addition, it is preferred if the sites in the AEP mRNA which are targeted by the antisense
10 oligonucleotides are sites in which mRNA secondary structure is not expected and at which proteins are not expected to bind.

The AEP-inhibiting antisense agents need not be perfectly complementary to an AEP gene or mRNA in order to be effective. Rather, some degree of
15 mismatches may be acceptable if the antisense oligonucleotide is of sufficient length. The oligonucleotides should have sufficient length and complementarity so as to selectively hybridise to an AEP transcript under physiological conditions. Preferably, mismatches are absent or minimal.

20 The AEP inhibitor, suitably, following administration to the patient, is able to contact and inhibit AEP at a suitable site in the patient. Although it is desirable for the AEP inhibitor to be cell permeant, it is believed that non-permeant AEP inhibitors may be useful since they may be taken into an appropriate cell by endocytosis.

25 An "effective amount" of the AEP inhibitor is an amount effective to modulate the immune response in the patient to a clinically useful extent.

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AEP is believed to be involved in processing of protein antigens which are destined to be loaded into and presented by Class II MHC molecules. In a preferred embodiment of the invention the patient to be treated has or is at risk of a disease which involves MHC Class II molecules. Although we
5 are not bound by any theory concerning the invention, we believe that AEP may play an important role in autoimmune disease because it may recognise and cleave at sites which are normally hidden by glycosylation at asparagine residues. Glycosylation at asparagine residues is common in mammalian proteins and glycosylated asparagine residues are not cleaved
10 by AEP. An abnormal reduction in asparagine glycosylation of a self protein may lead to it being susceptible to AEP cleavage at these cryptic sites and, therefore, the peptide produced from the abnormally glycosylated protein may be susceptible to loading into and presentation to the immune system by Class II MHC molecules. Bacterial proteins, such
15 as tetanus toxin, are typically not asparagine glycosylated and so, if they contain appropriate asparagine residues, are susceptible to degradation by AEP and presentation on Class II MHC molecules in any case.

Class II MHC molecules that remain associated with invariant chain
20 cannot bind other peptides. Therefore, inhibition of AEP may reduce the competency of class II MHC molecules for binding antigenic peptides and reduces the presentation of antigenic peptides by Class II MHC molecules.

Preferably, the diseases are autoimmune diseases and it is particularly
25 preferred if the patient to be treated has or is at risk of an autoimmune disease such as rheumatoid arthritis, insulin-dependent diabetes mellitus, multiple sclerosis, Hashimoto's thyroiditis, coeliac disease, myasthenia

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gravis, pemphigus vulgaris, systemic lupus erythromatosus and Grave's disease.

In further preferred embodiments of the invention the patient to whom the
5 inhibitor of AEP is administered has or is at risk of an allergic reaction or
has undergone, or is to undergo, an organ or tissue or cell transplant in
which graft rejection may ensue.

Several allergies and hypersensitivity reactions involve undesired
10 responses to harmless environmental antigens inhaled, ingested or
otherwise contacted. In several cases the antigens involved are known
and, in as much as CD4 T cells are activated by class II MHC/peptide
complexes, inhibition of processing of these antigens by AEP inhibitors is
believed to be likely to block or reduce their display to the T cells which
15 cause the allergic or hypersensitive reactions.

Typical allergies include:

Allergic rhinitis (hay fever) and allergic asthma induced by grass and tree
20 pollens, dust-mite faeces, cat/dog fur and so on; contact hypersensitivity
reactions induced by: metals (nickel, cobalt, copper, palladium),
pentadecacatechol (poison ivy) or bee venom; food allergies to shellfish,
milk, eggs, fish, wheat; and drug allergies eg to penicillin, aspirin.

25 Thus, the AEP inhibitors of the invention are useful in treating allergies
and allergic reactions and hypersensitivity reactions. In addition to those
allergies and hypersensitivity reactions mentioned above, it is
contemplated that the AEP inhibitors described herein are useful in

treating allergic or hypersensitivity conditions associated with any of the following: the feline skin and salivary gland allergen of the domestic cat *Felis domesticus* (the amino acid sequence of which is disclosed in WO 91/06571), the major protein allergens from the house dust mite *dermatophagoides* (amino acid sequences disclosed in WO 94/24281) and allergens present in any of the following: grass, tree and weed (including ragweed) pollens; fungi and molds; foods eg fish, shellfish, crab, lobster, peanuts, nuts, egg and milk; stinging insects eg bee, wasp and hornet and the chironomidae (non-biting midges); spiders and mites; mammals such as dog, horse, rat, guinea pig, mice and gerbil; latex; biological detergent additives; drugs eg penicillins and other antibiotics and anaesthetic agents.

More particularly allergies to insect proteins arise from proteins from the housefly, fruit fly, sheep blow fly, screw worm fly, grain weevil, silkworm, honeybee, non-biting midge larvae, bee moth larvae, mealworm, cockroach and larvae of *Tenibrio molitor* beetle. All of these insect allergens are of particular relevance to allergic problems arising in the workplace.

It will be appreciated that the AEP inhibitors may be used on their own, or that they may be administered in conjunction with other forms of treatment of the allergic or hypersensitivity conditions, which are known in the art.

Allografts (from same species, different individual) or xenografts (different species) are frequently rejected because of differences at the MHC genetic locus between the donor and the recipient. MHC matching improves the chances of graft acceptance as do immunosuppressive drug

regimes. Blockade or reduction in the presentation of peptides on class II MHC molecules using the inhibitors of AEP described herein may be useful in immunosuppression and may be used instead of or in conjunction with existing suppressors of T cell activation such as cyclosporin or FK506.

Tissue and organ transplants which may benefit from additional immunosuppressive methods include kidney, lung, heart, liver, cornea and bone marrow. Prior treatment of both the organ and host may be beneficial.

It will be appreciated that the AEP inhibitors may be used on their own, or that they may be administered in conjunction with other forms of immunosuppression such as those described above or as mixtures of AEP inhibitors or as mixtures of an AEP inhibitor with, for example, an inhibitor of cathepsin S as described below.

The aforementioned inhibitors for use in the invention or a formulation thereof may be administered by any conventional method including oral, parenteral (eg subcutaneous or intramuscular) injection topical and the like. The treatment may consist of a single dose or a plurality of doses over a period of time.

Whilst it is possible for an inhibitor for use in the invention to be administered alone, it is preferable to present it as a pharmaceutical formulation, together with one or more acceptable carriers. The carrier(s) must be "acceptable" in the sense of being compatible with the compound

of the invention and not deleterious to the recipients thereof. Typically, the carriers will be water or saline which will be sterile and pyrogen free.

The formulations may conveniently be presented in unit dosage form and
5 may be prepared by any of the methods well known in the art of pharmacy. Such methods include the step of bringing into association the active ingredient (inhibitor of AEP) with the carrier which constitutes one or more accessory ingredients. In general the formulations are prepared by uniformly and intimately bringing into association the active ingredient with
10 liquid carriers or finely divided solid carriers or both, and then, if necessary, shaping the product.

Formulations in accordance with the present invention suitable for oral administration may be presented as discrete units such as capsules, cachets
15 or tablets, each containing a predetermined amount of the active ingredient; as a powder or granules; as a solution or a suspension in an aqueous liquid or a non-aqueous liquid; or as an oil-in-water liquid emulsion or a water-in-oil liquid emulsion. The active ingredient may also be presented as a bolus, electuary or paste.

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A tablet may be made by compression or moulding, optionally with one or more accessory ingredients. Compressed tablets may be prepared by compressing in a suitable machine the active ingredient in a free-flowing form such as a powder or granules, optionally mixed with a binder (eg
25 povidone, gelatin, hydroxypropylmethyl cellulose), lubricant, inert diluent, preservative, disintegrant (eg sodium starch glycolate, cross-linked povidone, cross-linked sodium carboxymethyl cellulose), surface-active or dispersing agent. Moulded tablets may be made by moulding in a suitable

machine a mixture of the powdered compound moistened with an inert liquid diluent. The tablets may optionally be coated or scored and may be formulated so as to provide slow or controlled release of the active ingredient therein using, for example, hydroxypropylmethylcellulose in
5 varying proportions to provide desired release profile.

Formulations suitable for topical administration in the mouth include lozenges comprising the active ingredient in a flavoured basis, usually sucrose and acacia or tragacanth; pastilles comprising the active ingredient
10 in an inert basis such as gelatin and glycerin, or sucrose and acacia; and mouth-washes comprising the active ingredient in a suitable liquid carrier.

Formulations suitable for parenteral administration include aqueous and non-aqueous sterile injection solutions which may contain anti-oxidants,
15 buffers, bacteriostats and solutes which render the formulation isotonic with the blood of the intended recipient; and aqueous and non-aqueous sterile suspensions which may include suspending agents and thickening agents. The formulations may be presented in unit-dose or multi-dose containers, for example sealed ampoules and vials, and may be stored in a freeze-dried
20 (lyophilised) condition requiring only the addition of the sterile liquid carrier, for example water for injections, immediately prior to use. Extemporaneous injection solutions and suspensions may be prepared from sterile powders, granules and tablets of the kind previously described.

25 Preferred unit dosage formulations are those containing a daily dose or unit, daily sub-dose or an appropriate fraction thereof, of an active ingredient.

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It should be understood that in addition to the ingredients particularly mentioned above the formulations of this invention may include other agents conventional in the art having regard to the type of formulation in question, for example those suitable for oral administration may include flavouring agents.

It will be appreciated that the pharmaceutical composition of the invention may further comprise an agent which is usefully administered to the patient in need of modulation of their immune response. For example, the composition may further comprise an agent for treatment or prevention or amelioration of an autoimmune disease or allergic or hypersensitivity reaction. As a further example, the composition may further comprise an immunosuppressive agent such as cyclosporin or FK506.

It is particularly preferred if the AEP inhibitor is administered at or near to the site of the disease. For example, in the treatment of rheumatoid arthritis it is desirable for the AEP inhibitor to be administered at or close to the joints affected. In this case, the AEP inhibitor may be administered by topical application.

A further aspect of the invention provides the use of an inhibitor of asparaginyl endopeptidase in the manufacture of a medicament for modulating the immune response in a patient in need of such modulation.

A still further aspect of the invention provides use of an inhibitor of asparaginyl endopeptidase for modulating the immune response in a patient in need of such modulation.

Yet still further aspects of the invention provide an inhibitor of asparaginyl endopeptidase for use in medicine and a pharmaceutical composition comprising an inhibitor of AEP and a pharmaceutically acceptable carrier.

- 5 In addition to AEP (as described for the first time herein), two other classes of proteases are involved in the Class II MHC pathway. These are the papain family of cysteine proteases (cathepsins S, L, B and H), and the aspartic acid family (cathepsins D and E).
- 10 Recent results from cathepsin L, D and B knock-out mice suggest that they have no major effect, at least in peripheral antigen presenting cells. This suggests that either there is considerable redundancy or that these enzymes are not the really important enzymes. The results described herein show that AEP is an important enzyme in processing Class II MHC
- 15 antigens, and it is contemplated that the inhibitors of AEP when used alone may be useful. However, in some circumstances it may be desirable to administer to the patient, or combine in a pharmaceutical formulation or in a therapeutic system (kit of parts), an inhibitor of AEP with an inhibitor of one or more of these other enzymes which are involved in the Class II
- 20 MHC pathway. In particular, since cathepsin S appears to be an important enzyme in invariant chain processing (see, for example, WO 97/40066) it is believed that a combination of cathepsin S inhibitors and AEP inhibitors may be particularly useful.
- 25 Thus, combinations of AEP inhibitors as disclosed herein and inhibitors cathepsin D or cathepsin E are contemplated. Known inhibitors of aspartic acid enzymes such as cathepsin D and cathepsin E include, for

example, pepstatin and CGP 53437 (Ciba-Geigy; see Alteri *et al* (1993) *Antimic. Ag. Chemotherapy* 37, 2087-).

Similarly, combinations of AEP inhibitors and lysosomal cysteine protease inhibitors (ie inhibitors of cathepsin S, L, B and H) are contemplated. Broad specificity inhibitors of these enzymes include leupeptin and the epoxide compound E64 or E64D. However, it is contemplated that cathepsin S inhibitors (in particular cathepsin S-selective or specific inhibitors) may be particularly advantageously combined with AEP inhibitors. Cathepsin S inhibitors are described in detail in WO 97/40066, all of which are incorporated herein by reference. In particular, the following cathepsin S inhibitors are believed to be useful in combination with AEP inhibitors: morpholino-Leu-homophenylalanine-vinyl sulphone; Leu-Leu-Leu-vinyl sulphone with various N-terminal blocking groups; vinyl sulphones based on the invariant chain cleavage sites cut by cathepsin S.

It is preferred that combinations of invariant chain-based inhibitors targeted to both AEP and cathepsin S are used in combination. Combinations of such inhibitors may readily be devised by the skilled person by reference to the AEP inhibitors disclosed herein and by reference to the invariant chain-based cathepsin S inhibitors described in WO 97/40066. Examples of such combinations include, but are not limited to, BI-Glu-Asn-Leu-vinyl sulphone and/or BI-Leu-Arg-Met-vinyl sulphone (to block cathepsin S) plus one or more of BI-Ser-Gln-Asn; BI-Leu-Glu-Asn; BI-Leu-Gln-Asn; BI-Pro-Glu-Asn; and BI-Leu-Lys-Asn, all as vinyl sulphones (attached via the Asn residue).

As an alternative to vinyl sulphones as the reactive group, any of the reactive groups mentioned above ("Q") may be used in the inhibitors.

Other suitable combinations include invariant chain sequence based competitive inhibitors (as disclosed herein for AEP and as disclosed in WO 97/40066 for cathepsin S); and compounds disclosed herein as AEP inhibitors which are not based on the invariant chain in combination with any cathepsin S inhibitor.

The invention also includes a method of reducing the processing of a protein antigen by a MHC Class II molecule by a cell, the method comprising contacting the cell with an inhibitor of asparaginyl endopeptidase.

In one embodiment of the invention, an appropriate antigen presenting cell (APC), such as a dendritic cell or macrophage or B lymphocyte is transfected with a pool of cDNAs (or is contacted with proteins expressed from a pool of cDNAs) wherein the pool of cDNAs is believed to contain a cDNA encoding a protein which activates a T cell and may be associated with an autoimmune disease. The cDNA which encodes a protein which activates a T cell is selected and encodes a putative autoimmune protein. The assay may be carried out in the presence or absence of AEP inhibition in order to determine the involvement of AEP processing in the disease and also to determine any cryptic Asn glycosylation sites which may be important in the self-antigen immune response. Thus, the T cell is used to "read out" whether the cDNA for a putative autoantigen is being expressed and being presented, and whether AEP inhibitors have any effect on this.

A further aspect of the invention provides a method of identifying a compound for modulating Class II MHC antigen processing the method comprising contacting a test compound with asparaginyl endopeptidase and selecting a compound which reduces its activity.

This method (or screening assay) of the invention is suitably carried out in a format which allows for many test compounds to be screened simultaneously such as in a 96-well plate format. The activity of AEP is conveniently measured using any suitable substrate but it is preferred if the substrate is one which, upon cleavage by AEP, gives rise to a readily-detectable product. For example, the product may be coloured or fluorescent or detectable in some other way. It is particularly preferred if the product is fluorescent; a preferred substrate is Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide.

The test compound is preferably a compound from a library of test compounds. The compound may be one which has been made by synthetic chemistry methods (such as combinatorial chemistry methods) or it may be a naturally occurring compound. Without prejudice to the type of compounds which can usefully be screened (which include any suitable compounds), it is believed that many inhibitors of AEP will have a peptide or peptide-like structure. Thus, it is preferred if the test compound has a peptide or peptide-like structure.

It will be appreciated that the method or screening assay may also include a step for eliminating non-specific inhibitory compounds or for positively

selecting those inhibitors which inhibit AEP but do not substantially inhibit other cysteine proteases which do not have AEP activity.

In a preferred embodiment of the invention, any test compounds which are identified as being capable of reducing the activity of AEP are selected for a second screening step which involves assessing their capacity to influence Class II MHC molecule loading and presentation in a suitable cell.

Thus, preferably the second step is the determination of whether the compound is capable of substantially inhibiting the loading and presentation of peptides on an appropriate Class II MHC molecule-containing cell. A convenient way of determining this is to assess whether the compound is capable of substantially inhibiting T cell activation by an appropriate Class II MHC molecule-containing cell. This may be done as described in the Example.

A further aspect of the invention comprises a non-human transgenic animal wherein a gene encoding asparaginyl endopeptidase has been modified and the animal expresses substantially no asparaginyl endopeptidase from said gene.

By "transgenic" animal we specifically include animals in which all or part of a gene have been "knocked out" or otherwise made substantially incapable of expressing an asparaginyl endopeptidase. Suitable mice can be made using standard methodology involving, for example, genetic manipulation of embryonic stem (ES) cells as is well known in the art. A cDNA encoding AEP is described in Chen *et al* (1997) *J. Biol. Chem.*

272. 8090-8096, and this information may be used in designing and making the transgenic animals of the invention.

It is particularly preferred if the non-human animal is a mouse, but it may
5 be any suitable non-human animal including rat, rabbit and the like.

In a preferred embodiment of the invention, the non-human transgenic animal which expresses substantially no AEP from an AEP gene comprises a genetic background which predisposes the animal to an autoimmune disease either spontaneously or upon administration of protein antigen. Animals, especially mice, with a suitable genetic background are well known, for example NOD mice, which are a model for diabetes, EAE (experimental autoimmune encephalomyelitis) mice which are a model for allergic encephalomyelitis and CIA mice which are a model for collagen-induced arthritis. The mice used for the model disease EAE are of the MHC haplotypes H-2u or H-2k (eg PL/J or B10.PL strains). The mice used for the model disease CIA are of the MHC haplotype H-2q (eg DBA/1J strain). Such mice are described or referenced in Cantorna *et al* (1998) *J. Nutr.* **128**, 68-72; Xiao & Link (1997) *Clin. Immunol. Immunopathol.* **85**, 119-128; and Kumar *et al* (1997) *J. Exp. Med.* **185**, 1725-1733.

Alternatively, the animal will be transgenic for a human Class II MHC molecule that predisposes to a human autoimmune disease.

In a further preferred embodiment, the non-human transgenic animal which expresses substantially no AEP from an AEP gene is further transgenic for a human Class II MHC molecule and, optionally, further

transgenic for CD4. Mice with these further characteristics are described in Altmann *et al* (1995) *J. Exp. Med.* 181, 867-875; Yamamoto *et al* (1994) *J. Ex. Med.* 180, 165-171; and Woods *et al* (1994) *J. Exp. Med.* 180, 173-181.

5

It will be appreciated that non-human animals which have combinations of characteristics can be made by crossing appropriate animals. Thus, the animals of the preferred embodiments of the invention may be made by crossing suitable animals.

10

From the foregoing it will be appreciated that new AEP inhibitors are disclosed.

15

A further aspect of the invention provides an inhibitor of asparaginyl endopeptidase which has the structure $Bl-(X_a-X_n)-Asn-Q$ wherein Bl is any suitable N terminal blocking group; X_a-X_n are the n amino acid residues immediately N terminal to an Asn cleavage site in the invariant chain of Class II MHC molecules; Asn is an asparagine residue; and Q is a group capable of reacting with the active site of asparaginyl endopeptidase.

20

Suitable blocking groups, amino acids, and groups capable of reacting with the active site of AEP are the same as disclosed earlier in the application. It is preferred if the group capable of reacting with the active site of AEP can react with the active site cysteine residue.

25

The n amino acid residues N terminal to an Asn cleavage site can readily be determined by reference to the sequence of the invariant chain and by reference to the Asn residue number as described earlier. Typically the

number of amino acid residues is between 1 and 25; preferably, it is between 2 and 10; more preferably between 2 and 5.

Preferred inhibitors include BI-Ser-Gln-Asn-Q; BI-Leu-Glu-Asn-Q; BI-Leu-Glu-Asn-Q; BI-Pro-Glu-Asn-Q; BI-Leu-Lys-Asn-Q; BI-Gln-Asn-Q; BI-Glu-Asn-Q; BI-Asp-Glu-Asn-Q; BI-Asn-Gly-Asn-Q; BI-Phe-Pro-Asn-Q; BI-Val-Pro-Asn-Q; and BI-His-His-Asn-Q where Q is any reactive group as defined earlier and BI is any N-terminal blocking group as defined earlier.

10

A further aspect of the invention provides an inhibitor of asparaginyl endopeptidase which has the structure (Xb-Xc)Asn(Xd-Xe) wherein (Xb-Xc) are the r amino acid residues immediately N terminal to an Asn cleavage site in the invariant chain of Class II MHC molecules and (Xd-Xe) are the s amino acid residues immediately C terminal to an Asn cleavage site in the said invariant chain; Asn is an asparagine residue; and r and s are independently between 2 and 25.

Preferably, r and s are independently between 2 and 10; more preferably r and s are independently between 2 and 5.

20

The invention will now be described by reference to the following Examples and Figures wherein

Figure 1: Processing of TTCF by a leupeptin-insensitive cysteine endopeptidase activity. (a) TTCF was digested *in vitro* in the presence of disrupted lysosomes (1-2 μ g) in the presence or absence of iodoacetamide (1 mM), leupeptin, E64 or pepstatin (each at 0.1 mg/ml). Digestions

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- were in 0.1M NH_4 acetate at pH 4.5 or Na_2HPO_4 , pH 6.0 or 7.0. After 4 hours at 37°C, the reactions were separated by Tris-tricine SDS-PAGE and stained with Coomassie Blue. (b) Cleavage sites: TTCF digestion products were separated by SDS PAGE and electrophoretically transferred to nitrocellulose membrane. Individual fragments numbered 1-6 were subjected to 5 cycles of Edman degradation. N-terminal sequence obtained is shown in bold following upstream residues from TTCF (tetanus toxin numbering). The N-terminal sequence of His-tagged TTCF was obtained for fragments 1 and 3. (c) Residues Asn 1184 and Glu 1184 in TTCF were mutated to alanine (see Methods) and digestions performed as before. The mutation abolishes cleavage at the mutated site but not at the first site (doublet at 47 kD). In this experiment cleavage of the third site was minimal.
- Figure 2:** TTCF is processed by an asparaginyl endopeptidase. (a) Chromatography of the TTCF processing activity on Mono-S resin. Fractions eluted by NaCl gradient were incubated with the substrates Z-Ala-Ala-Asn-NHMec or Z-Phe-Arg-NHMec in the presence or absence of E64 (trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane). E64 had no inhibitory effect on the cleavage of Z-Ala-Ala-Asn-NHMec (not shown). (b) Fractions as above were incubated with 10 μg TTCF in the presence or absence of E64 for 4 hours at 37°C prior to SDS-PAGE analysis. (c) Each fraction was analysed by SDS-PAGE and Western blotting. Electrophoretically transferred material was probed with an affinity purified antiserum raised against the peptide KGIGSGKVLKSGPQC from human legumain¹.

Figure 3: Peptide inhibitors of AEP block asparaginyl endopeptidase activity and TTCF processing *in vitro* and *in vivo*. (a) TTCF was digested with purified AEP in the presence or absence of 0.2 mg/ml of the peptides indicated. Peptides were N and C-terminally blocked by not removing
5 Fmoc or amide groups respectively. (b) Purified AEP or a crude lysosomal protease mixture were incubated with Z-Ala-Ala-Asn-NHMec, Z-Phe-Arg-NHMec (cathepsins L&B) or Z-Val-Val-Ala NHMec (cathepsin S) in the presence of increasing concentrations of AENK or AEQK. Release of 7-amino-4-methyl coumarin was measured after 10,
10 20 or 30 minutes. (c) Peripheral blood mononuclear cells were isolated from donor A.K. and preincubated for 15 minutes in the presence or absence of 1 mg/ml AENK (■) or AEQK (○) prior to addition of TTCF antigen. At different times the cells were washed, fixed in 0.05% glutaraldehyde and co-cultured with autologous TTCF specific T cell
15 clones.

Figure 4 shows the structure of various AEP inhibitors.

Figure 5 shows the structure of various protease inhibitors which can be
20 converted to AEP inhibitors as described in the text.

Figure 6 shows that AEP can cleave the Class II MHC invariant chain (both p31 and p41 isoforms).

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Example 1: Asparaginyl endopeptidase is involved in processing and class II MHC presentation of a microbial antigen

Summary

5

Foreign antigens must be proteolytically processed to allow loading of peptides onto class II MHC molecules. To investigate which proteases might be involved we exposed a domain of the microbial antigen, tetanus toxin (TTCF) to disrupted lysosomes purified from a human B cell line.

10 Surprisingly, the dominant processing activity was not one of the known lysosomal cathepsins but rather an asparagine specific cysteine endopeptidase which we call AEP. This enzyme appears similar if not identical to a mammalian homologue of legumain¹, an asparaginyl endopeptidase found originally in plants and parasites^{2,3}. We designed
15 competitive peptide inhibitors of AEP which specifically block its asparaginyl endopeptidase activity and inhibit processing of TTCF *in vitro* and its presentation *in vivo* to T cells. Since N-glycosylation renders asparagine resistant to cleavage by AEP we suggest that this enzyme may represent a further example of the ability of the innate immune system to
20 focus its attention on microbial non-self.

To analyse processing of a foreign antigen without making prior assumptions about which enzymes are involved we exposed a 47 kD domain of the tetanus toxin antigen (TTCF) to disrupted lysosomes
25 isolated from the human B cell line EDR. As shown in Fig 1a, the antigen was fragmented to produce a discrete series of products at pH 4.5 but not at pH 7.0 and minimally at pH 6.0, confirming the lysosomal origin of the protease(s) involved (Fig 1a). Surprisingly, we could not

inhibit lysosomal digestion of TTCF with leupeptin or E64(trans-epoxysuccinyl-L-leucylamido-(4-guanidino)butane), broad spectrum inhibitors of lysosomal cysteine proteases or with pepstatin, an inhibitor of the aspartic acid cathepsins E and D (Fig 1a). LHVS, a more specific inhibitor of cathepsin S⁴ was also without effect (not shown). Nor could we reproduce the lysosomal TTCF digestion pattern or indeed generate any of these fragments using purified cathepsins L, S, B, D or E, all of which have been implicated in the class II MHC antigen processing pathway (reviewed in refs 5 & 6). Further inhibitor studies revealed however that the activity was sensitive to iodoacetamide (Fig 1a), to N-ethyl maleimide and to high concentrations of the diazomethane, Z-Phe-Phe-CHN₂ (not shown) indicating that one or more cysteine protease(s) were nonetheless involved.

We sequenced each major TTCF digestion products to gain more information on this processing activity. All visible fragments arose from 3 cleavages in the TTCF protein and each occurred after an asparagine residue (Fig 1b). Mutation of asparagine 1184 and glutamic acid 1185 to alanine completely abolished cleavage at this site (Fig 1c) indicating that one (presumably the asparagine) or both of these residues is crucial for processing.

These results suggested that B cell lysosomes contain one or more novel cysteine endopeptidase(s) with possible specificity for asparagine. Although not previously described in antigen presenting cells, an enzyme with similar properties called legumain has been found in the seeds of leguminous plants^{2,3}, in *Schistosoma mansoni*³ and recently in some mammalian tissues such as kidney and placenta¹. We next partially

purified the TTCF processing activity from a crude B cell lysosome fraction to establish whether or not it was an asparaginyl endopeptidase. As shown in Figure 2b, a peak of TTCF processing activity eluted from a cation-exchange resin at 0.4M NaCl. This activity co-eluted precisely
5 with an activity capable of cleaving the substrate Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide substrate (Fig 2a). Another protease activity, presumably cathepsin L and/or B, capable of cleaving the substrate Z-Phe-Arg-NHMec, partially overlapped the asparaginyl endopeptidase activity (Fig 2a). However this activity was completely inhibited by E64 (Fig 2a)
10 while both the TTCF and Z-Ala-Ala-Asn-NHMec processing activity were unaffected (Fig 2b) confirming that a novel cysteine endopeptidase was involved. To compare this activity to the recently described mammalian form of legumain we generated anti-peptide antisera to several regions of the mammalian preprolegumain sequence¹. Blotting of each fraction with
15 an antisera to residues 125-140 (see methods) revealed the presence of a protein with the same mobility (35 kD) as purified pig kidney legumain which co-eluted with the TTCF and peptide substrate processing activity (Fig 2c). Finally, exactly the same processing sites were recognised by the crude lysosomal fraction, the partially purified TTCF processing
20 activity and purified pig kidney legumain (Figs 1b & 2b, ref 1 and data not shown). Thus, mammalian legumain or a closely related enzyme, is present in human B lymphocytes and *in vitro*, is the major enzyme responsible for processing this antigen. We propose the name AEP (Asparaginyl endopeptidase) for this enzyme to distinguish it from the
25 plant enzyme.

We decided to test the possibility that high concentrations of asparagine containing peptides might act as competitive inhibitors. As shown in Fig

3. the N and C-terminally blocked tetrapeptide Ala-Glu-Asn-Lys-NH (AENK) substantially inhibited the processing of TTCF by AEP (Fig 3a). Lys-Asn-Asn-Glu-NH (KNNE) also inhibited but was less effective. Importantly, the same concentrations of the glutamine analogues AEQK and KQKE did not inhibit TTCFr processing demonstrating specific competitive inhibition of AEP (Fig 3a). To establish that inhibition of AEP was specific, we measured the hydrolysis of different synthetic peptide substrates in the presence and absence of these tetrapeptides. As shown in Fig 3b, AENK completely blocked cleavage of Z-Val-Ala-Asn-NHMe by B cell lysosomal fractions but had no effect on the hydrolysis of the cathepsin L/B substrate, Z-Phe-Arg-NHMe or on the Cathepsin S substrate Z-Val-Val-Arg-HNMe.

If AEP plays a role in TTCF processing in antigen presenting cells then sufficiently high levels of the competing AENK peptide might be expected to interfere with normal class II MHC loading of TTCF epitopes. Following a 15 minute preincubation with or without tetrapeptides, freshly isolated peripheral blood mononuclear cells (PBMC) were incubated with TTCF antigen in the continued presence or absence of the peptides. At different times, the cells were washed, fixed and then co-cultured with different autologous T cell clones to assess expression of different peptide/MHC complexes. Presentation to most clones was detectable after 60 minutes of antigen pulsing in the absence of competing peptide but in the presence of AENK, presentation to some clones was either undetectable (AK 20 and AK 90; Fig 3b) or profoundly inhibited (eg AK 6). At later times there was some recovery of presentation of these epitopes as expected for a competitive inhibitor. Several controls ruled out the possibility that the inhibitory effect of the AENK competitor is

simply due to non-specific toxicity. First, presentation to other clones recognising other regions of TTCF was not affected by the AENK competitor (eg AK 111, 71 and 33: Fig 3c). Secondly, there was a clear differential sensitivity to AENK versus AEQK (Fig 3c), strongly suggesting that specific inhibition of AEP was responsible for the slowed kinetics of presentation. Thirdly, the inhibitory effects of AENK could be overridden by using TTCF pre-digested *in vitro* by AEP. In other words, the requirement for AEP *in vivo*, and hence the inhibitory effect of AENK, was by-passed by AEP cleavage *in vitro* (data not shown). Taken together our results reveal AEP as a new and highly specific processing activity in the class II MHC pathway. Preliminary studies show that AEP is present in a variety of other antigen presenting cell types.

Although we uncovered this processing activity using an antigen proteolysis assay, it is possible that it also plays a role in invariant chain processing as well. Splenocytes from cathepsin D gene targeted mice continued to produce invariant chain processing products even when all known cysteine protease activity were also suppressed by leupeptin⁷. Villadangos *et al* suggested that other non-cysteine proteases (besides cathepsin D) must be initiating Ii (invariant chain) cleavage. Cathepsin E is one possible candidate but so too is AEP since, although it is a cysteine protease, it is completely resistant to leupeptin (Fig 1a). Intact p31 Ii is readily cleaved by AEP *in vitro* and we are currently assessing the possibility that AENK also interferes with Ii processing. However, the differential effect of the AENK inhibitor on T cell clones and the fact that pre-digestion of antigen with AEP abolished the inhibitory effect of the AENK inhibitor argues that its primary inhibitory effect in our studies was on TTCF processing.

Can any special significance be attached to the existence of an asparaginyl endopeptidase in antigen presenting cells? One possibility is that microbial proteins might in general be better substrates than host proteins since the latter could resist AEP attack by N-glycosylation of particularly sensitive asparagine residues. It is very striking that while TTCF is an extremely good substrate for AEP, cleavage occurs at only 3 of its 47 asparagine residues. This suggests that there may be additional determinants of AEP specificity to be identified and that strategic N-glycosylation might give substantial protection. Thus the innate immune systems ability to bind microbial antigen through recognition of non-self molecular 'patterns', might be harnessed to an 'innate' bias towards microbial antigen processing as well. In addition, our data raise the possibility that disturbances of N-glycosylation might result in altered processing of self proteins by AEP leading to presentation of 'cryptic' epitopes with possible pathological consequences.

Methods

TTCF processing activity. Lysosomal fractions from the human B cell line EDR were prepared on 27% Percoll density gradients as previously described⁸ using ¹²⁵I-labelled transferrin (internalised for 30 minutes at 37°C) and β -hexosaminidase to identify endosomal/plasma membrane and lysosomal fractions respectively. The peak of lysosomes was collected by centrifugation at 40,000 g for 1 hour, removed from the underlying Percoll pellet, collected again at 50,000 g for 1 hour and stored frozen. For separation of leupeptin-insensitive cysteine protease activity, 7.5×10^9 cells were homogenised in a ball-bearing homogenizer and nuclei and

unbroken cells removed by centrifugation at 2,000 g for 10 minutes. A membrane pellet was collected at 40,000 g and solubilised in 50 mM citrate buffer, pH 5.5 containing 0.1% CHAPS (3-((cholamidopropyl)dimethylammonio]-1-propane-sulphonate). The extract
5 was centrifuged at 2,000 g for 20 minutes and then applied to a Mono-S column (Pharmacia). Fractions were eluted with a gradient of NaCl and monitored for TTCF processing activity.

Proteins and peptides. A histidine tagged derivative of the C terminal
10 domain of tetanus toxin was prepared^{9,10} and purified¹⁰ as described. This protein has residues 872-1315 of the complete toxin (1-1315) preceded by the sequence MGHGHHHHHHHHHHSSGHIEGRHI. Mutagenesis of residues 1184/5 was performed using template EH106 obtained by cloning the His-TTCF (Reference 10) as an XbaI/BamHI fragment into pSL1180
15 (Pharmacia). Site directed mutagenesis of residues 1184/85 was performed according to the method of Mikaelian and Sargent¹¹ using the mutagenic primer CGC TAC ACT CCG AAC GCG GCG ATC GAT TCT TTC GTT and flanking primers M13 rev (AGCGGATAACAATTTACACAGGA) and M13 seq
20 (GTAAAACGACGGCCAGT). After sequencing to confirm mutagenesis, recloned back into pET16B for expression. Tetrapeptides were synthesised using Fmoc chemistry leaving the C-terminus amidated and the N-terminus retaining the Fmoc group. LHVS was a kind gift from Hidde Ploegh, MIT.

25

Antigen presentation. Peripheral blood mononuclear cells were prepared by Ficoll/Paque centrifugation and used fresh. T cell clones specific for TTCF were established from donor A.K. according to¹². Epitope

Example 2: Asparaginyl endopeptidase can process the Class II MHC invariant chain

20 A key step in the generation of peptide/class II MHC complexes is the complete removal of a protein called the invariant chain from the class II MHC $\alpha\beta$ dimer. This is a dedicated chaperone for class II MHC which must be removed by proteolysis before any peptide can be loaded (Cresswell (1996) *Cell* 84, 505-). Normally, its removal requires, first, 25 proteolysis to produce a small fragment (known as CLIP – about 3 kD) which still blocks the class II peptide binding site, and second, the action of a protein called DM which removes this peptide. Blocking invariant chain removal effectively shuts down loading of all other peptides. There

are two alternatively spliced forms of the invariant chain usually known as p31 and p41. p41 is minor in most cells except dendritic cells where it accounts for around 40% of invariant chain.

- 5 *In vitro*, cathepsin S can produce the small fragment (CLIP) suitable for DM action (Riese *et al* (1996) *Immunity* 4, 357-) but it is likely that other enzymes are involved in invariant chain processing in living cells. For example, inhibitors of cathepsin S result in the accumulation of an invariant chain fragment considerably larger (~ 10 kD) than CLIP. This
10 shows that cathepsin S is only really essential at the later stages of invariant chain processing and that other enzymes can produce the 10 kD fragment. It has now been found that AEP can degrade the invariant chain *in vitro*. Thus, AEP processing of the invariant chain may initiate its destruction in some or all cells (some cells, eg thymic cortical epithelial
15 cells do not seem to express cathepsin S).

- The human EBV-transformed lines PALA or AL-EBV were radiolabelled for 15 minutes at 37°C with 0.5 mCi/ml ³⁵S-Translabel (ICN), washed and lysed in Tris-buffered saline containing 1% Triton X-100 plus
20 protease inhibitors (Davidson & Watts (1989) *J. Cell. Biol.* 109, 85). The lysates were cleared by centrifugation and pre-cleared with Protein G sepharose. Invariant chain/class II MHC complexes were precipitated with the VIC-Y1 antibody (gift from W Knapp). The complexes were collected on Protein G sepharose, washed 3 times in Tris-buffered saline,
25 1% Triton X-100 and resuspended in 50 mM citrate buffer, 1 mM EDTA, 5 mM dithiothreitol with or without 0.05% CHAPS (Calbiochem). Purified aspariginyl endopeptidase was added to 5 mU per 20 µl reaction. Digestions were performed at 37°C for 30 minutes and analysed by Tris-

Circumstantial evidence has previously shown that enzymes, other than cathepsin S, L, B and D, can degrade the invariant chain in living cells. This comes from the finding that in spleen cells from a cathepsin D knock-out mouse also treated with leupeptin (which inhibits cathepsins S, L and B), the early stages of invariant chain processing still proceed (Villadangos *et al* (1997) *J. Exp. Med.* 186, 549-). The results presented here suggest that AEP could be responsible for this residual invariant chain processing activity.

References for Example

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CLAIMS

1. A method of modulating the immune response in a patient in need of such modulation, the method comprising administering to the patient an effective amount of an inhibitor of asparaginyl endopeptidase.
5
2. A method according to Claim 1 wherein the patient has or is at risk of a disease which involves MHC Class II molecules.
- 10 3. A method according to Claim 1 or 2 wherein the disease is an autoimmune disease.
4. A method according to Claim 3 wherein the disease is rheumatoid arthritis.
- 15 5. A method according to Claim 1 or 2 wherein the patient has or is at risk of an allergic or hypersensitivity reaction.
6. A method according to Claim 1 or 2 wherein the patient has
20 undergone or is to undergo a transplant.
7. A method according to Claim 6 wherein the material transplanted, or to be transplanted, has been contacted with an effective amount of an inhibitor of asparaginylendopeptidase.
25
8. A method according to any one of the preceding claims wherein the inhibitor is a competitive inhibitor.

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9. A method according to Claim 8 wherein the competitive inhibitor is a peptide comprising is an asparagine-containing peptide.
10. A method according to Claim 9 wherein the peptide is an N and C-terminal blocked peptide Ala-Glu-Asn-Lys-NH (AENK) or Lys-Asn-Asn-Glu-NH (KNNE).
11. A method according to Claim 1 to 6 wherein the inhibitor is a non-competitive or irreversible inhibitor.
12. A method according to Claim 11 wherein the inhibitor has the structure $Bl-(X)_n-Asn-Q$ where Bl is any suitable N terminal blocking group; X is an amino acid residue; n is between 1 and 100, Asn is an asparagine residue and Q is a group capable of reacting with the active site cysteine of asparaginyl endopeptidase.
13. A method according to any one of the preceding claims further comprising administering to the patient an effective amount of an agent for treatment or prevention or amelioration of an autoimmune disease or an allergic or hypersensitivity reaction.
14. A method according to any one of Claims 1 to 12 further comprising administering to the patient an effective amount of an immunosuppressive agent.
15. A method of reducing the processing of a protein antigen by a MHC Class II molecule by a cell, the method comprising contacting the cell with an inhibitor of asparaginyl endopeptidase.

16. A method according to Claim 15 wherein the inhibitor is a competitive inhibitor.
17. A method according to Claim 16 wherein the competitive inhibitor is a peptide comprising an asparagine-containing peptide.
18. A method according to Claim 17 wherein the peptide is an N and C-terminal blocked peptide Ala-Glu-Asn-Lys-NH (AENK) or Lys-Asn-Asn-Glu-NH (KNNE).
19. A method according to Claim 15 wherein the inhibitor is a non-competitive or irreversible inhibitor.
20. A method according to Claim 19 wherein the inhibitor has the structure $Bl-(X)_n-Asn-Q$ where Bl is any suitable N terminal blocking group; X is an amino acid residue; n is between 1 and 100, Asn is an asparagine residue and Q is a group capable of reacting with the active site cysteine of asparaginyl endopeptidase.
21. A method according to any one of Claims 15 to 20 wherein the cell is, or is comprised in a tissue or organ, for transplantation into a patient.
22. Use of an inhibitor of asparaginyl endopeptidase in the manufacture of a medicament for modulating the immune response in a patient in need of such modulation.

23. Use according to Claim 22 wherein the patient has or is at risk of a disease which involves MHC Class II molecules.

24. Use according to Claim 22 or 23 wherein the disease is an autoimmune disease.

25. Use according to Claim 24 wherein the disease is rheumatoid arthritis.

26. Use according to Claim 22 or 23 wherein the patient has or is at risk of an allergic or hypersensitivity reaction.

27. Use according to Claim 22 or 23 wherein the patient has undergone or is to undergo a transplant.

28. Use according to any one of Claims 22 to 27 wherein the inhibitor is a competitive inhibitor.

29. Use according to Claim 28 wherein the competitive inhibitor is a peptide comprising is an asparagine-containing peptide.

30. Use according to Claim 29 wherein the peptide is an N and C-terminal blocked peptide Ala-Glu-Asn-Lys-NH (AENK) or Lys-Asn-Asn-Glu-NH (KNNE).

31. Use according to any one of Claims 22 to 27 wherein the inhibitor is a non-competitive or irreversible inhibitor.

32. Use according to Claim 31 wherein the inhibitor has the structure $Bl-(X)_n-Asn\ Q$ where Bl is any suitable N terminal blocking group; X is an amino acid residue; n is between 1 and 100, Asn is an asparagine residue and Q is a group capable of reacting with the active site cysteine of asparaginyl endopeptidase.

33. Use according to any one of Claims 22 to 32 wherein the patient is administered an effective amount of an agent for treatment or prevention or amelioration of an autoimmune disease or an allergic or hypersensitivity reaction.

34. Use according to any one of Claims 22 to 32 wherein the patient is administered an effective amount of an immunosuppressive agent.

35. Use of an inhibitor of asparaginyl endopeptidase for modulating the immune response in a patient in need of such modulation.

36. Use of an inhibitor of asparaginyl endopeptidase for reducing the processing of a protein antigen by a MHC Class II molecule by a cell.

37. An inhibitor of asparaginyl endopeptidase for use in medicine.

38. A pharmaceutical composition comprising an inhibitor of asparaginyl endopeptidase and a pharmaceutically acceptable carrier.

39. A pharmaceutical composition according to Claim 38 further comprising an agent which is usefully administered to a patient in need of modulation of the immune response.

40. A pharmaceutical composition according to Claim 38 further comprising an agent for treatment or prevention or amelioration of an autoimmune disease or an allergic or hypersensitivity reaction.

5

41. A pharmaceutical composition according to Claim 38 further comprising an immunosuppressive agent.

42. A pharmaceutical composition comprising an inhibitor of asparaginyl endopeptidase, an inhibitor of cathepsin S and a pharmaceutically acceptable carrier.

10

43. A method of identifying a compound for modulating Class II MHC antigen processing the method comprising contacting a test compound with asparaginyl endopeptidase and selecting a compound which reduces its activity.

15

44. A method according to Claim 43 wherein the activity of asparaginyl endopeptidase is measured using a substrate which upon cleavage by said endopeptidase, yields a readily detectable product.

20

45. A method according to Claim 44 wherein the substrate is Z-Ala-Ala-Asn-7-(4-methyl)coumarylamide and the product is fluorescent.

46. A method according to any one of Claims 43 to 45 the method further comprising the step of determining whether the so selected compound is capable of substantially inhibiting the loading and

25

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47. A method according to Claim 46 wherein it is determined whether the so selected compound is capable of substantially inhibiting T cell activation by an appropriate Class II MHC molecule-containing cell.

49. A non-human transgenic animal according to Claim 48 which is a mouse.

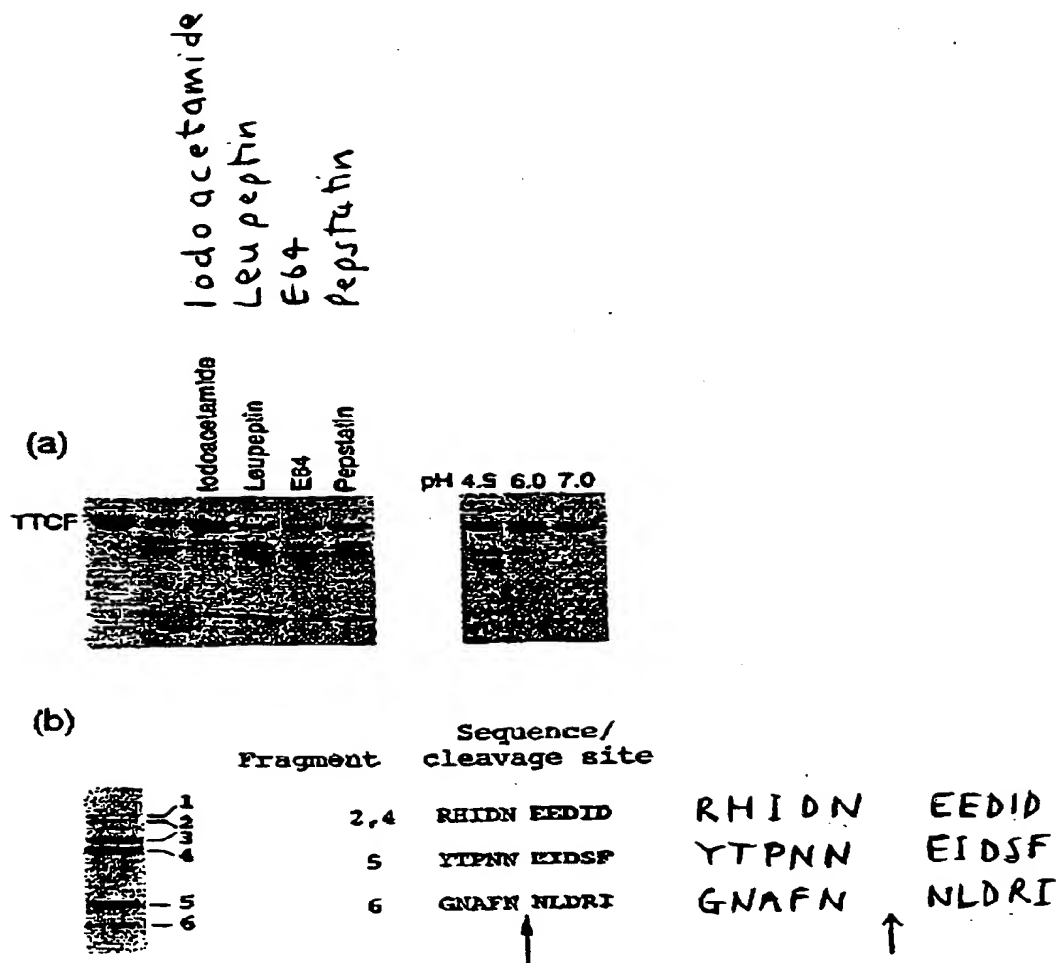
51. A non-human transgenic animal according to Claims 48 or 49 further transgenic for a human Class II MHC molecule and, optionally, further transgenic for human CD4.

52. An inhibitor of asparaginyl endopeptidase which has the structure BI-(X₁-X_n)-Asn-Q wherein BI is any suitable N terminal blocking group; X₁-X_n are the n amino acid residues immediately N terminal to an Asn cleavage site in the invariant chain of Class II MHC molecules; Asn is an

A method of reducing the processing of a protein antigen by a MHC Class II molecule by a cell, the method comprising contacting the cell with an inhibitor of asparaginyl endopeptidase.

SECRET

Fig 1



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Fig 1c

TTCF TTCF
(1184/5 NE) (1184/5 AA)

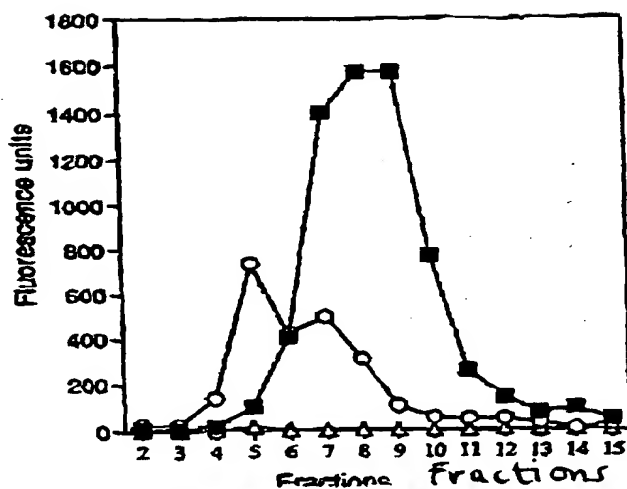
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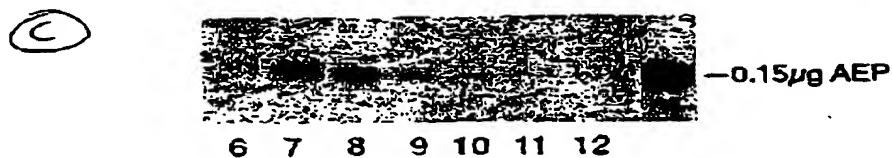
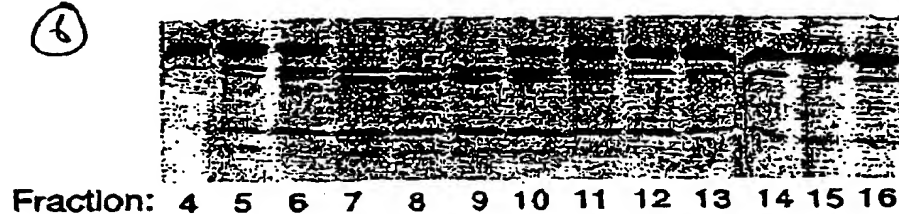
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Fig 2

363259 Fluorescence Unit



—■— Z-Ala-Ala-AsnNHMe Z-Ala-Ala-AsnNHMe
 —○— Z-Phe-Phe-NHMe Z-Phe-Phe-NHMe
 —△— Z-Phe-Phe-NHMe + E64 Z-Phe-Phe-NHMe + E64

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Fig 3a

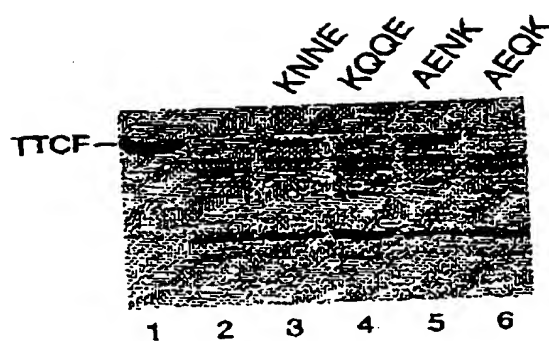
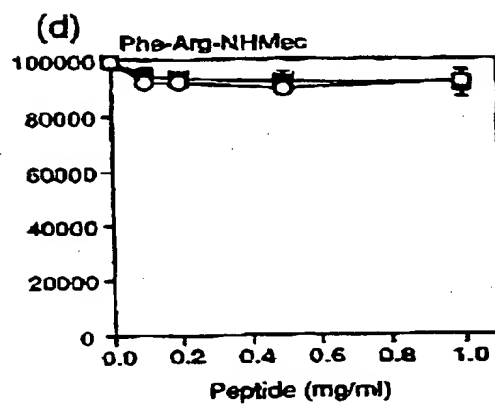
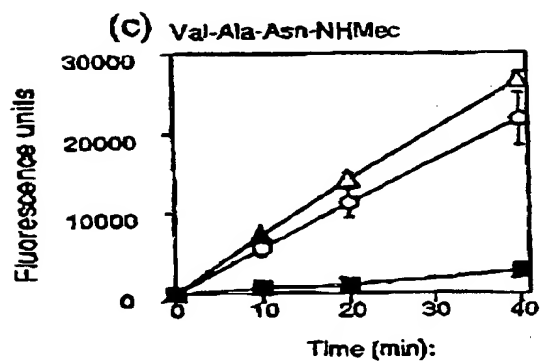
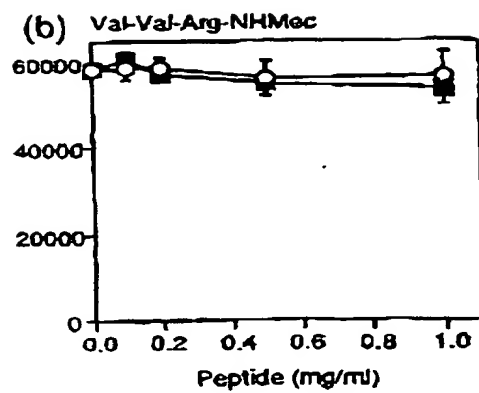
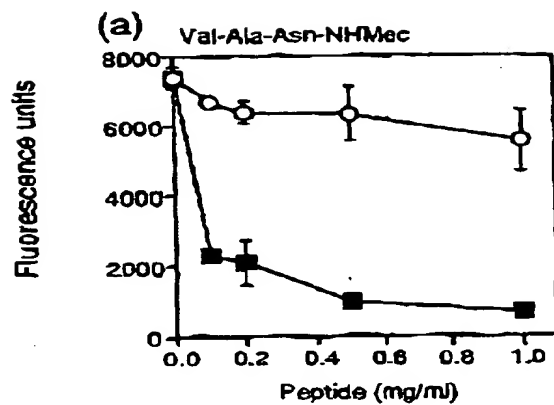
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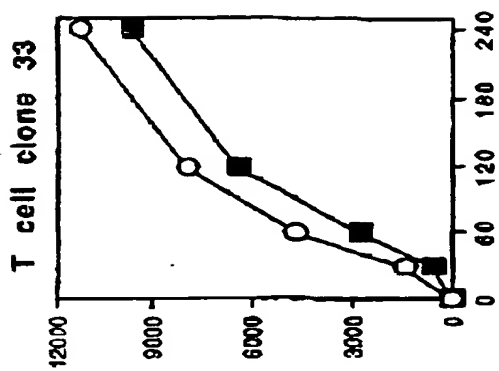
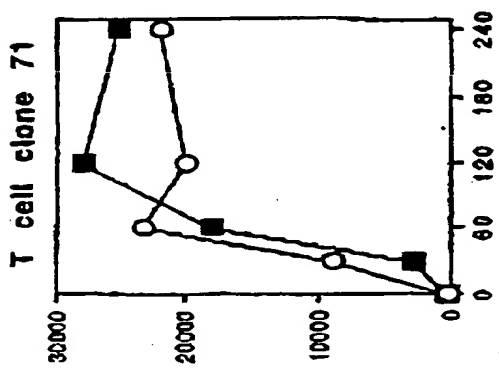
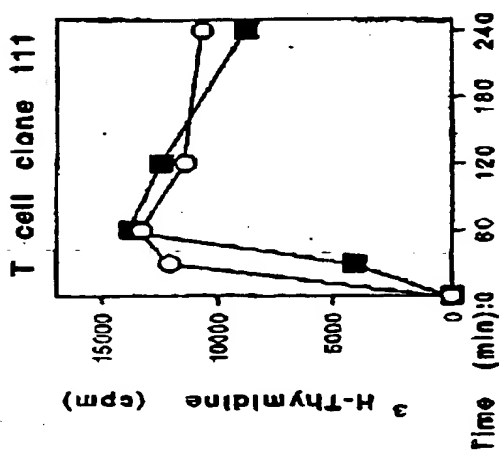
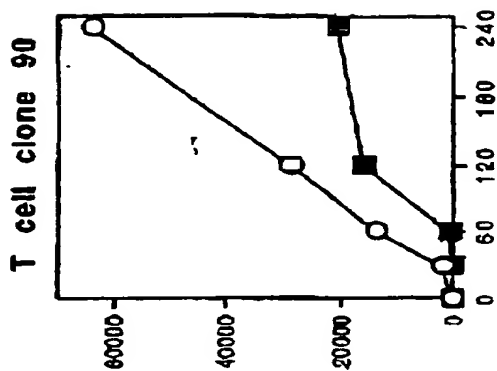
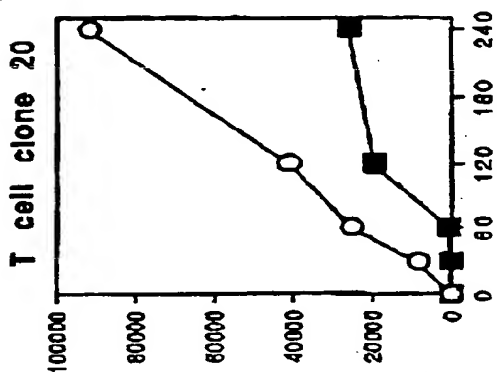
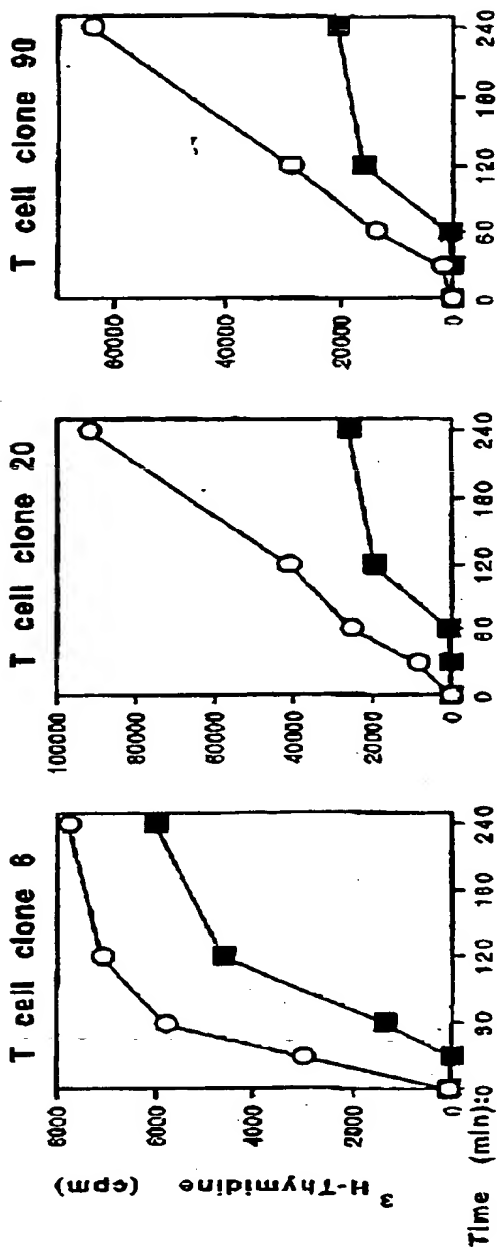
Fig 36

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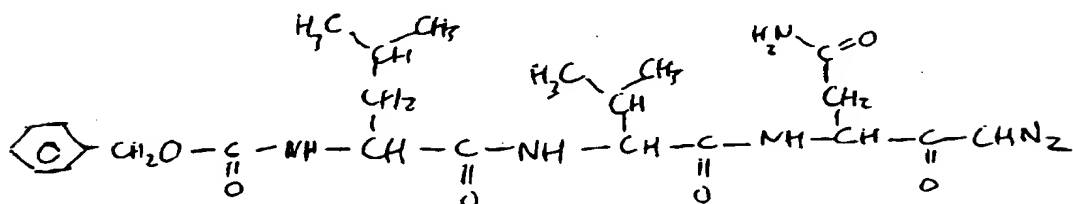


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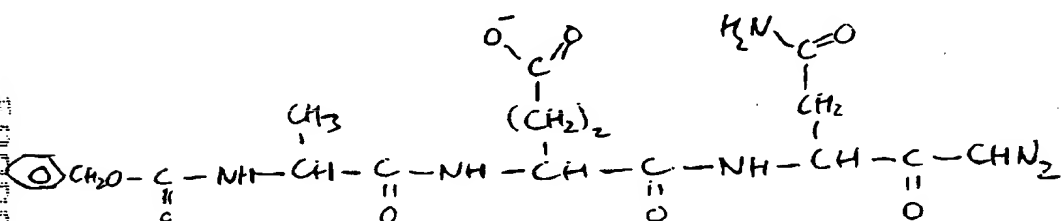
Figure 4 (page 2 of 6)

Peptidyl diazomethanes (refs 3,4) (have the general structure: R-C(=O)CHN₂)

(i) benzyloxycarbonyl-L-ucyl-valyl-asparaginyl-diazomethane

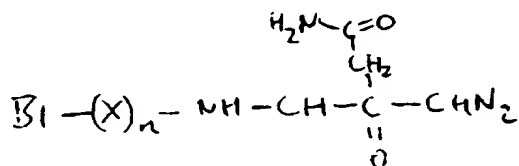


(ii) alanyl-glutamyl-asparaginyl diazomethane



(iii) Z-(X)_n-asparaginyl-diazomethane

Where BI = acetyl or benzyloxycarbonyl and X = any amino acid



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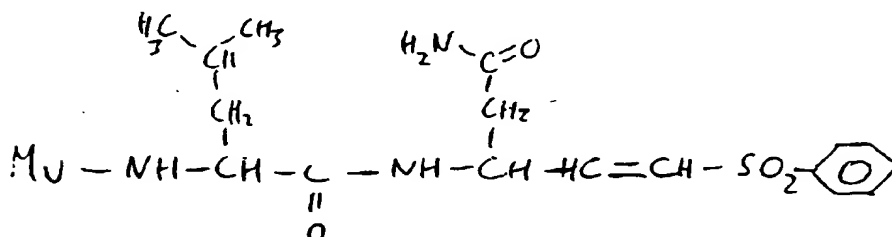
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Peptide vinyl sulph n s (ref 6)

(i) Morpholinurea-I ucyI-asparaginyI-vinylsulphone-phenyl

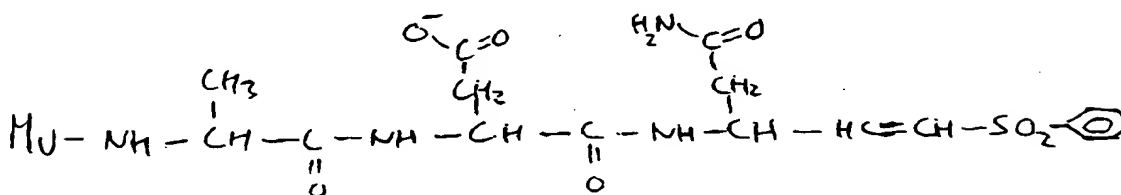
(Acetyl or benzyloxycarbonyl can replace morpholinurea)



MU = morpholinurea

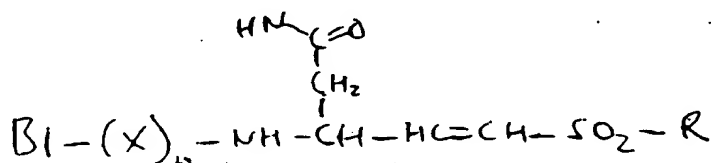
(ii) Morpholinurea-alanyI-glutamyl-asparaginyI-vinylsulphone-phenyl

(Acetyl or benzyloxycarbonyl can replace morpholinurea)



(iii) BI-(X)_n-asparaginyI -vinylsulphone-R

Where BI= N-terminal blocking group (acetyl, morpholinurea or benzyloxycarbonyl, X= any amino acid and R=alkyl or aryl terminating group.



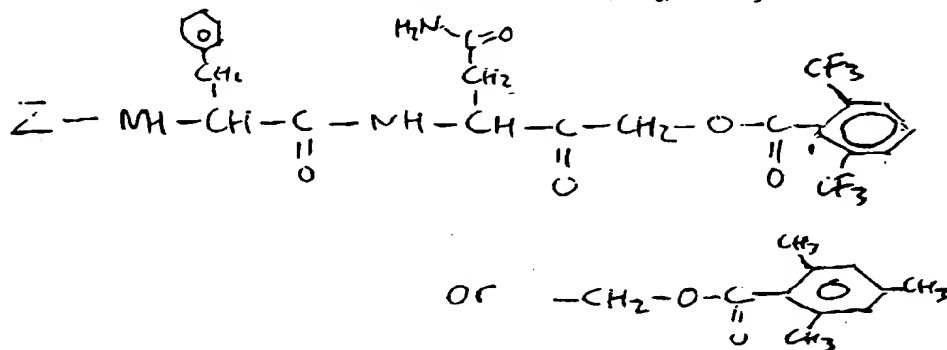
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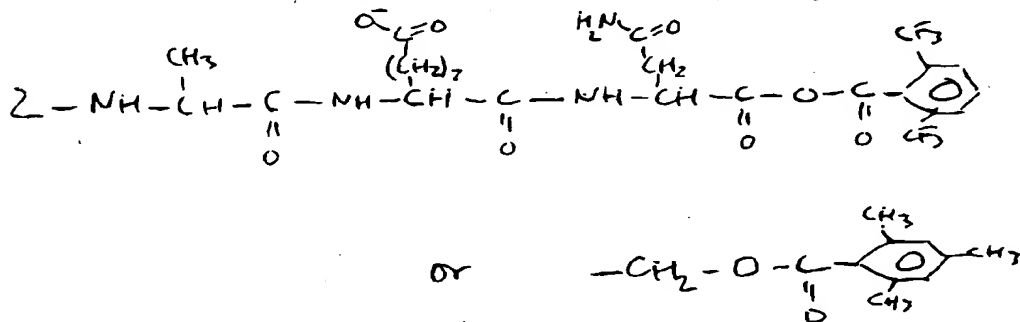
Figure 4 (page 4 of 6)

Peptidyl (acyloxy) methanes (ref 7)

- (i) Z-Ph nyalanyl-asparaginy-CH₂OCO-[2,6-(CF₃)₂Phenyl
(ii) Z-Phenyalanyl-asparaginy-CH₂OCO-[2,4,6-(CH₃) Phenyl



- (iv) Z-alanyl-glutamyl-asparaginy-CH₂OCO-[2,4,6-(CH₃) Phenyl



- (iv) Z-(X)n-asparaginyl- $\text{CH}_2\text{OCO-R}$

Where X=any amino acid and R=[2,6-(CF₃)₂Phenyl or [2,4,6-(CH₃) Phenyl or other acyloxy methane group

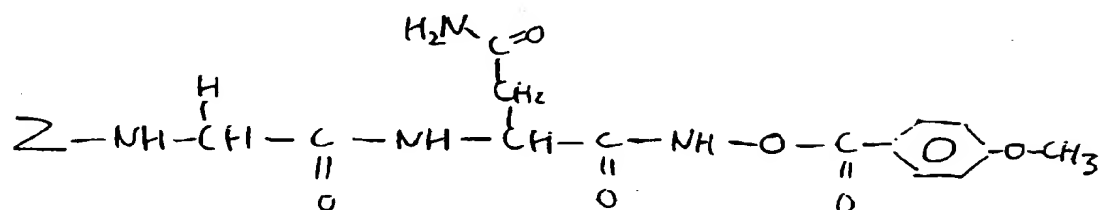
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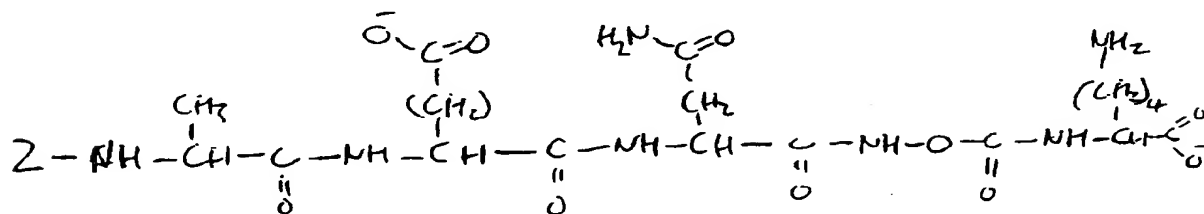
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Figure 4 (page) of 0

N,O-diacyl hydroxamates (ref 8)

(i) Z-Glycyl-asparaginyl-NHO-benzoyl(4-OCH₃)



(ii) Z-alanyl-glutamyl-asparaginy-NHO-CO-lysine-NH



(iii) Z-(X)_n-asparaginyl-NHO-CO-R

Where Z= benzyloxycarbonyl or other blocking group, X= any amino acid, and R= any O-acyl group.

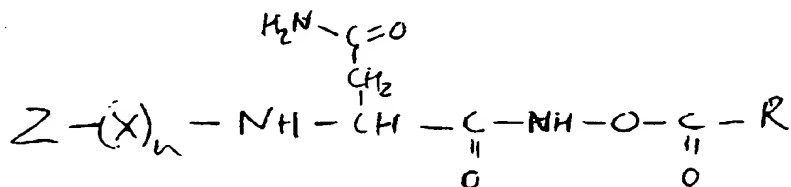
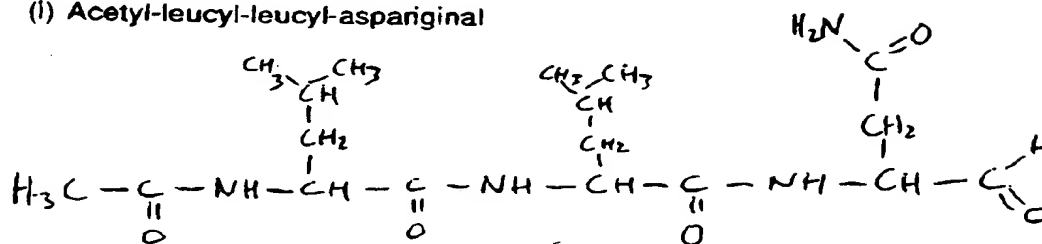


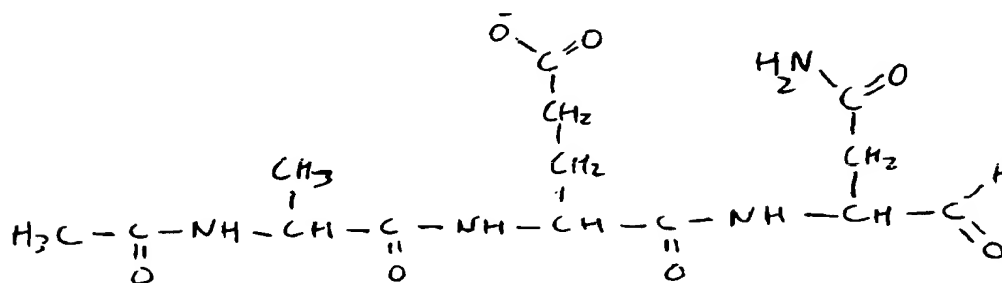
Figure 4 (page 6 of 6)

Peptide aldehydes (refs 1 & 2) :

(i) **Acetyl-leucyl-leucyl-aspariginal**



(ii) **Acetyl-alanyl-glutamyl-aspariginal**



(iii) Acetyl (or other blocking group)-(X)_n-Asparaginyl

—where X denotes any amino acid(s) in peptide linkage.

Elastatinal also blocks AEP. A more specific variant would be:

(iv)

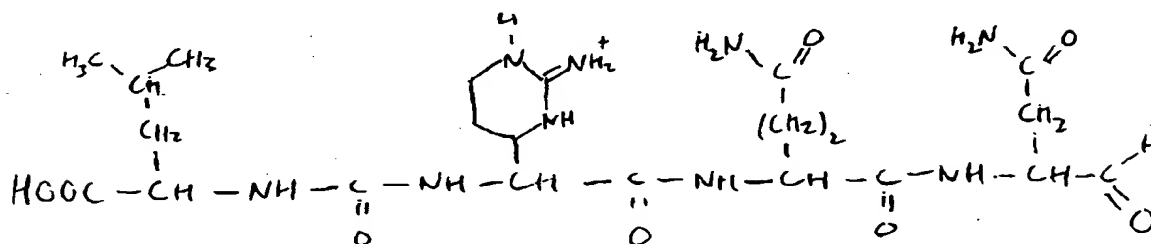
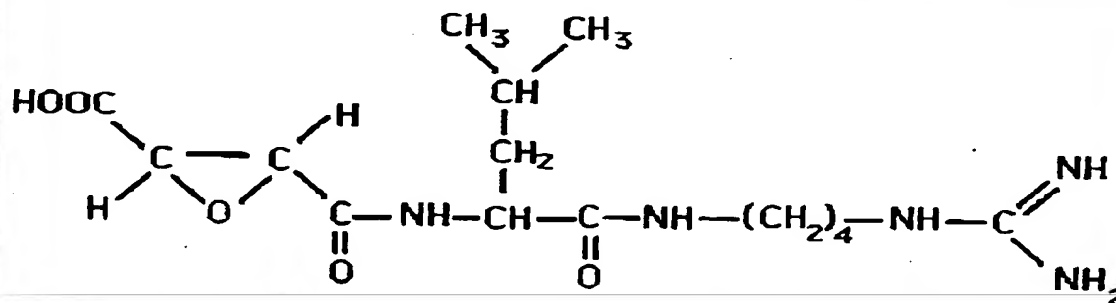


Figure 5 (page 1 of 6)

**E-64**

Structure: L-trans-epoxysuccinyl-leucylamide-(4-guanido)-butane or N-[N-(L-trans-carboxyoxiran-2-carbonyl)-L-leucyl]-agmatine



Inhibition spectrum: Irreversible inhibitor of cysteine proteinases. Does not inhibit serine proteinases.

Mechanism of action: Forms a thioether bond with the thiol of the active cysteine (1:1 enzyme/inhibitor molar ratio)

Properties: E-64 is soluble in aqueous solutions (stock solution 1 mM). Stable from pH 2.0 to pH 10.0 but unstable in ammonia or strong acids, which destroy the epoxide ring. Excellent active-site titrant of cysteine proteinases. Originally isolated from the culture medium of a mold, *Aspergillus japonicus*.
MW: 357.4

Suggested final concentration: 1-10 μ M



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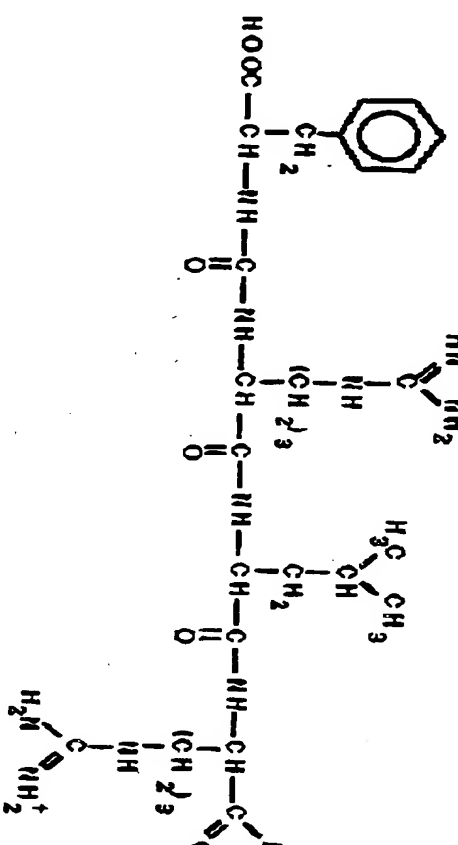
Figure 5 (page 2 of 6)

Leupeptin	
Structure: Acetyl-leucyl-leucyl-arginal	
 <chem>CC(=O)N[C@@H](C(C)C)C(=O)N[C@@H](C(C)C)C(=O)N[C@@H](CNC(=N)N)CC=O</chem>	
Inhibition spectrum: inhibits serine (trypsin ($K_i=13\text{ }\mu\text{M}$), plasmin, porcine kallikrein) and cysteine proteinases (papain, cathepsin B). Does not inhibit chymotrypsin and thrombin.	
Mechanism of action: Competitive and reversible inhibitor. Inhibition may be relieved by an excess of substrate.	
Properties: Soluble in water, ethanol, acetic acid and DMF (Stock solution: 10 mM) MW: leupeptin: 426.6; leupeptin hemisulphate monohydrate: 542.7	
Suggested final concentration: 1-10 μM (0.5-1 $\mu\text{g/ml}$)	

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Antipain	
Structure: [(S)-1-Carboxy-2-Phenyl]-carbamoyl-Arg-Val-arginal	
Inhibition spectrum: Inhibits papain, trypsin and plasmin to a lesser extent. More specific for papain and trypsin than leupeptin. The inhibitory potency of antipain is 100-fold higher than that of elastinal.	
Mechanism of action: Formation of a hemiacetal adduct between the aldehyde group of the inhibitor and the active serine of the proteinase.	
Properties: Soluble in H ₂ O, methanol and DMSO (Stock solution: 10 mM). Stable at -20° C. MW: 604.7 for antipain; 677.6 for antipain dihydrochloride	

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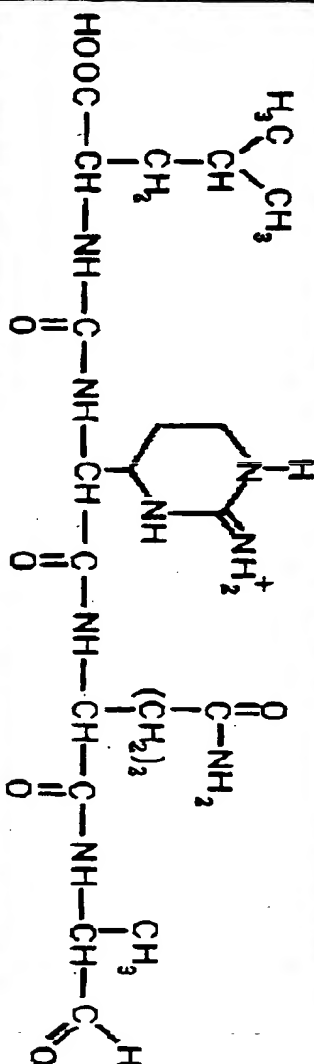
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Elastatinal

Structure: Leu-(Cap)-Gln-Ala-al,
N-[(S)-1-carboxy-isopentyl]-carbamoyl-alpha-(2-iminohexahydro-4(S)-pyrimidinyl)-
L-glycyl-L-glutaminyl-L-alaninal



Inhibition spectrum: Inhibits elastase-like serine proteinases ($K_i = 0.24 \mu\text{M}$ for porcine pancreatic elastase, $K_i = 50-80 \mu\text{M}$ for human leukocyte elastase). Specific for elastases, does not inhibit chymotrypsin or trypsin.

Mechanism of action: Formation of a hemiacetal adduct between the aldehyde group of the inhibitor and the active serine of the proteinase.

Properties: Soluble in water (Stock solution: 10 mM)
MW: 512.6

Suggested final concentration: 10-100 μM

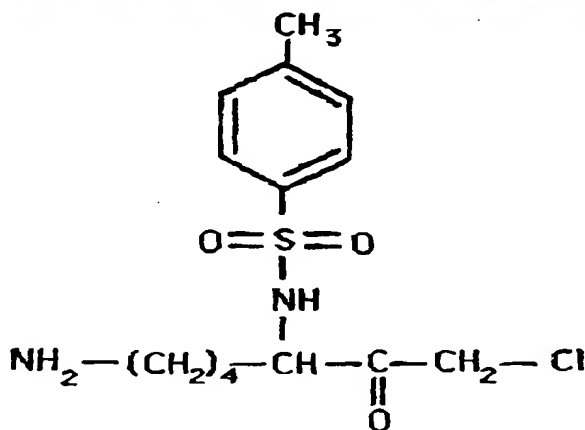
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TLCK

Structure: Tosyl Lysyl ChloromethylKetone:
1-Chloro-3-tosylamido-7-amino-2-heptanone



Inhibition spectrum: Irreversible inhibitor of trypsin. Also inhibits some serine proteases (kallikrein, thrombin, plasmin) and some cysteine proteases such as papain, bromelain or ficin. Does not inhibit chymotrypsin and zymogens.

Mechanism of action: Requires an active enzyme and forms a covalent bond with His of the active site. Does not react with zymogens so addition of TLCK in a crude extract inactivates only active protease. Does not react with protease-inhibitor complexes.

Properties: TLCK salts are soluble in water up to 20 mg/ml. More stable at pH less or equal to 6.0.
Soluble up to 5 mg/ml in methanol or to 1 mg/ml in NaCl 0.15 M.
MW: 369.3 (TLCK.HCl)

Suggested final concentration: 50 µg/ml



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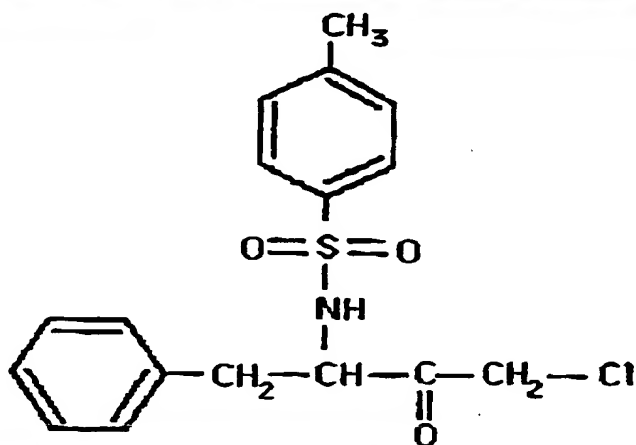
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TPCK

Structure: Tosyl Phenylalanyl ChloromethylKetone:
1-Chloro-3-tosylamido-4-phenyl-2-butanone



Inhibition spectrum: Irreversible inhibitor of chymotrypsin. Also inhibits some proteases cleaving substrates with a Phe at position P1 and some cysteine proteases such as papain, bromelain or ficin. Does not inhibit trypsin and zymogens.

Mechanism of action: Requires an active enzyme and forms a covalent bond with His of the active site. Does not react with zymogens so addition of TPCK in a crude extract inactivates only active protease. Does not react with protease-inhibitor complexes.

Properties: TPCK is soluble in ethanol up to 20 mg/ml but poorly in water. It is more stable at slightly acidic or neutral pH. It is destroyed in less than 30 min. at pH 9.0.
MW: 351.9

Suggested final concentration: 100 µg/ml



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Figure 6

